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14. ABSTRACT

The project combined a range of complex elements that all needed to succeed to produce an optimal result, ie, a sensitive radiation bioassay. The specialized cell line provided a reliable and consistent biological test target model. The cell synchrony experiments provided reliable data and demonstrated the ability to precisely separate G1 cells from asynchronous populations of L5178Y S/S cells. Data from cell flow cytometry (CFC) confirmed the successful elutriation and yield of narrow distributions of DNA content in G1 cells. Results also demonstrated the ability of the centrifugal elutriation technique to separate out populations of cells in later stages of the cell cycle. This offers a valuable tool for the design of enhanced sensitivity test systems that may need to examine the effects of stressors on specific susceptible stages in the cell cycle. Careful and rigorous analysis of the application of the comet assay for DNA single strand breaks found the results to be inconsistent when using the established routine laboratory protocol. Inconsistencies in the data for DNA content acquired by the comet assay and the effect on calculation of tail moment parameter reduced our level of confidence in this part of the assay. Our study identified a major problem that could not be resolved within the time and resource constraints of the current project. These findings give cause for some concern regarding claims for sensitivity of the comet assay technique and the status of publications in this area of DNA damage. There may be good reason to doubt the reliability of data given in some publications on comet assays that do not disclose information on DNA content or valid zero-dose values. It is apparent that most researchers are either unaware of the limitation or choose to ignore it. Considering the range of technique-dependent artifacts that we have identified it is questionable if meaningful comparisons of data can be made for comet assay analyses from different laboratories

15. SUBJECT TERMS

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UNITED STATES AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

ASIAN OFFICE OF AEROSPACE RESEARCH AND DEVELOPMENT

INTERNATIONAL RESEARCH CONTRACT REPORT:

EVALUATION OF A RADIO-SENSITIVE DNA BIOASSAY

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CSIRO Telecommunications and Industrial Physics, Sydney, Australia

AFOSR/AOARD CONTRACT Agreement # 902

INTERNATIONAL COLLABORATIVE SCIENTIFIC RESEARCH PROJECT: EVALUATION OF A RADIO-SENSITIVE DNA BIOASSAY.

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Telecommunications & Industrial Physics

12 October, 1999

Re: Agreement No. 902 – DNA Bioassay for Biological Effects of RF Radiation

This report describes the work carried out on an international collaborative research project funded through the US Air Force Office of Scientific Research/Asian Office of Aerospace Research and Development.

The research contract has given valuable support to the development of an effective collaborative partnership between research laboratories CSIRO in Australia and those associated with the USAF.

This final report (item no. 0001AB) is submitted under the terms of agreement of the research contract.

Dr. Stanley B Barnett, PhD
Senior Principal Research Scientist

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SUMMARY

The major goals of this research project were to;

* establish an effective international and multi-disciplinary research team,
* undertake preliminary study as a basis for the development of major grant applications for co-operative research,
* systematically develop a novel sensitive bioassay as a test for physical stressors including radiation,
* rigorously examine the suitability and adaptability of an existing "comet assay" technique as a possible test parameter.

The goals and objectives were achieved and the outcome was a good example of scientific co-operation.

The project combined a range of complex elements that all needed to succeed to produce an optimal result, ie, a sensitive radiation bioassay. The specialised cell line provided a reliable and consistent biological test target model. The cell synchrony experiments provided reliable data and demonstrated the ability to precisely separate G1 cells from asynchronous populations of L5178Y S/S cells. Data from cell flow cytometry (CFC) confirmed the successful elutriation and yield of narrow distributions of DNA content in G1 cells. Results also demonstrated the abilitiy of the centrifugal elutriation technique to separate out populations of cells in later stages of the cell cycle. This offers a valuable tool for the design of enhanced sensitivity test systems that may need to examine the effects of stressors on specific susceptible stages in the cell cycle.

Careful and rigorous analysis of the application of the comet assay for DNA single strand breaks found the results to be inconsistent when using the established routine laboratory

protocol. Inconsistencies in the data for DNA content acquired by the comet assay and the effect on calculation of tail moment parameter reduced our level of confidence in this part of the assay. Our study identified a major problem that could not be resolved within the time and resource constraints of the current project. These findings give cause for some concern regarding claims for sensitivity of the comet assay technique and the status of publications in this area of DNA damage. There may be good reason to doubt the reliability of data given in some publications on comet assays that do not disclose information on DNA content or valid zero-dose values. It is apparent that most researchers are either unaware of the limitation or choose to ignore it. Considering the range of technique-dependent artefacts that we have identified it is questionable if meaningful comparisons of data can be made for comet assay analyses from different laboratories.

This international collaborative research project has developed the essential groundwork for a continuing effective research program. An opportunity now exists to increase the sensitivity of the assay, particularly for low dose radiation. However, full exploitation of this tool requires a dedicated effort through a longer term collaboration than was available under the constraints the present arrangement. Because of the agreed importance of this project and the effective international scientific partnership that has developed, the research partners plan to submit further applications for funding to allow the research to develop and realize its full potential. This would be most effectively achieved by installing the essential equipment in the USAF Academy, Colorado Springs (currently under negotiation) where the Principal Investigator will work as a visiting scientist. During a period of 6 months the bioassay would be refined and used to quantify the effects other radiation stressors, such as microwave, laser or ultrasound radiation.

OBJECTIVES

- Establish an effective international collaborative scientific venture with the USAF Research Laboratory.
- Demonstrate the capability to undertake international collaborative research involving a multidisciplinary team from a number of research centres. The scientists involved in this proposal combine essential expertise in specialised areas of biological research.
- Develop and characterise a sensitive biosensor that can be reliably used (in subsequent research) to test for the effects of various physical stressors and radiations, including radio-frequency radiation, on the DNA in mammalian cells.
- Systematically examine the suitability of a specialised murine leukemia synchronised cell (L5178Y S/S) line as a radio-sensitive indicator of biological effects on DNA.
- Optimise the centrifugal elutriation technique to provide consistent separation of synchronised G1 cell fractions.
- Use the single cell micro-gel electrophoresis "comet assay" protocol in conjunction with an image analysis system to quantify changes in the distribution of single strand breaks of DNA in single cells.
- Quantify DNA content and distribution in populations of L5178Y S/S cells and compare data for comet assay and cell flow cytometry techniques.
- Compare data against a standard, unsynchronised cell population, e.g. lymphocytes in G_0 phase in the cell cycle.

RATIONALE

Data from research on bioeffects of Radio-Frequency Radiation (RFR) suggest the existence of biological responses at levels of exposure that do not produce significant increase in temperature. However, many of these reports are unsubstantiated. This is due largely to absence of organised systematic approach to research on bioelectromagnetics and to the absence of recognised sensitive, yet robust, experimental endpoints. There is a clear need to provide a reliable and sensitive biological indicator so that a solid data-base can be established for the formation of acceptable international standards for RF exposure (European Commission report 1996). There are continuing disagreements due to uncertainty about the existence of significant non-thermal bioeffects that may result from low-level long-term exposure to RFR in telecommunications (Barnett 1994). Publications of variable scientific quality have accumulated a substantial database describing biological responses to radio-frequency radiation at specific absorption rates too low to involve thermal mechanisms of interaction (McKinlay 1997). Exposures from military equipment, particularly the Ultra Wide Band, represent a significant risk of producing bioeffects because of the very high power contained in short pulses of microwave emissions. Any effect of such radiation that interferes with DNA is potentially damaging to human health. If human cells are continually damaged in a way that causes changes in their DNA without killing the cells, this can lead to the development of diseases such as cancer. Expert groups have reiterated the need for organised collobarative research on effects of low level electromagnetic fields (Repacholi 1998). It is anticipated that the proposed biological test system will have wide application for various physical stressors.

Recent studies have reported DNA breakages in brain tissue (Lai and Singh 1995, 1996) following acute exposure to microwave radiations at moderate field strengths, ie, close to the IEEE/ANSI standards limit for safe human exposure. The consequence of DNA strand breaks is uncertain but it can lead to disruption of normal cell functions and onset of disease such as cancer. The literature on DNA effects of non-ionizing microwave radiation is equivocal with different studies using variations of experimental procedures and assays. The primary objective of the present study is to apply improved design to ensure optimum sensitivity and repeatibility of the biological test system, and to establish the level of exposure to ionizing radiation that produces single strand DNA breaks. The validity of the test procedures themselves will be carefully examined.

It is only through the development of a consistently repeatable test system that reliable scientific data can be collected and assessed for the development of relevant international standards for safe exposure to non-ionizing forms of radiation, such as microwave and RF radiation.

Most published studies on biological effects of non-ionizing radiation (NIR) evaluate data from asynchronous cell populations. The results of such studies are used as a basis for the assessment of safety of radio-frequency radiation (RFR). However, there is fundamental problem which seriously limits the sensitivity of such data. "Normal" cells have two major periods of DNA repair (G1 and late-S-G2); this makes data on DNA damage from asynchronous cell populations (as typically used in studies of gene toxicology) relatively difficult to interpret because of nearly simultaneous DNA repair. In normal cells, rapid repair can easily eliminate low levels of DNA damage during irradiation or during post-irradiation handling, even at low temperatures. The L5178Y S/S cell line, derived from

mouse leukemia lymphoblasts, lacks the ability to repair DNA damage in the G1 phase of its cell cycle. Thus, the potential damaging effect of physical stressors will be maximised when synchronous populations are irradiated.

The rationale is to undertake a systematic study that;

- increases the sensitivity of the biological endpoint,
- tests for internal consistency and sensitivity of the specially sensitive bioassay by comparison with normal mammalian cells, asynchronous G_0 lymphocytes,
- tests against X-ray, known to adversely affect DNA repair,
- demonstrates suitability of synchronised cell system as a sensitive indicator of biological effects of radiation,
- provides an *in vitro* cell model that would have widespread application as a biological sensor of various stressors including both ionizing and non-ionizing radiation.

AIMS

This study will exploit an important property of the radio-sensitive L5178Y S/S cells; their deficiency in DNA repair in a specific stage, G1, of cell division/growth. It is hypothesised that populations of the cell line, exposed to stressors during the G1 phase, may be ultra-sensitive to radiation-induced DNA damage.

In order to provide a reliable biosensor it is necessary to;

- characterise growth patterns of synchronised L5178Y S/S cells and obtain qualitative and quantitative data on DNA damage,
- standardize techniques for cell synchronisation, cell culture, DNA alkaline hydrolysis, single cell microgel electrophoresis, DNA "comet" assay and image analysis,
- test the sensitivity and consistency of the test system by exposure to X-ray, a known mutagen.

STUDY PROTOCOL

1. Cell Culture Maintain normal exponential growth
2. Monitor Cell Concentration of Asynchronous Cell Population Distribution
3. Select Optimum Population of Asynchronously growing L5178Y S/S Cells;

   Analyse Distribution with Coulter Channeliser & Software
4. Calibrate Centrifugal Elutriation Procedures
5. Collect Fractions of specific cells sizes at Various Rotor Speeds
6. Confirm DNA Content in Collected Fractions by Cell Flow Cytometry
7. Select Optimum G1 Fraction for Synchrony Experiments
8. Irradiate G1 Cells and Positive Control (G_0) Fresh Lymphocytes
9. Single Cell Gel (SCG), Alkaline Hydrolysis, Electrophoresis, Propidium Iodide Stain
10. DNA spread in gels and ready for immediate Comet acquisition
11. DNA Fluorescence, "Comet" Microscopic Image
12. Data Acquisition, Excel Files of Comet Data Parameters
13. Image Analysis, customised software
14. Comet Assay, Tail Moment (Tail Length x %DNA in Tail)
15. DNA Content, Analysis By Sigma-Plot
16. Compare DNA Content for Comet Assay and Flow Cytometry
17. Dose Response Analysis

METHODS

Cell growth was synchronised prior to irradiation to ensure that cells were exposed in the G1 phase of division when deficient in DNA repair, thereby maximising the sensitivity of the biological test system. This also avoids possible errors due to differing sensitivity in each stage of the cell cycle. The spread of single strand breaks (ssb) in DNA following alkaline hydrolysis and exposure to an electrophoretic field was quantified using computerised image analysis of the DNA "comet" tail moment following exposure to increasing doses of X-irradiation, a known clastogenic physical agent.

CELL CULTURE

The radiosensitive murine lymphoma cell line, L5178Y S/S was isolated by Beer *et al.* (1963), and Lett *et al.* (1964). The first S stands for sensitive (as in radiosensitive) and the second for simplified (as in simplified growth medium containing bovine serum (Cohn) fractions II, IV-4 and V, rather than full bovine serum). Nagasawa *et al.* (1980) showed that there was a deficiency in DNA repair capability in the G1 phase of the cell cycle for the S/S cells.

Culture protocol

Populations of L5178Y S/S cells are maintained by dilution in 10-fold increments (37$\pm$ 0.5° C, pH equilibrated medium) when their density reaches 5-7 x10^5 cells/ml; since the cells grow in suspension, trypsin treatment is unnecessary and no "lag time" results when cells are subcultured. Optimal growth is maintained by diluting populations 2-3 times per week. The growth medium is Fischer's supplemented with Cohn serum fractions II, IV-4 and V plus penicillin, streptomycin and neomycin and appropriate phosphate buffers;

the full culture medium has a slightly lower pH than typical tissue culture media, but cells are grown in standard tissue culture incubators with a 5% CO_2 atmosphere (Beer *et al.* 1963; Lett *et al.* 1964).

To maintain consistency in growth and survival characteristics the cells are diluted to a concentration of $0.1\text{-}1x10^5$ per ml. When cell growth reaches too high a concentration (e.g., 10^6 per ml) cells aggregate into large clumps of multicellular spheroids. The cell size is also influenced by cell concentration; at high concentrations cells become smaller. These factors can introduce erroneous data as cell cycle phase is estimated on the basis of cell diameter. In order to achieve consistency of cell counts and good fraction separation a constant cell concentration was maintained.

Cell Counts/Coulter Channeliser

Cell growth was monitored by regular microscopic examination and cell count. A Coulter system (Coulter model ZBI) was used to perform counts on diluted samples of cell suspensions and the use of a Coulter Channeliser provided essential information on the distribution of populations of different cell cycle fractions. Software from Coulter was used for data analysis and graphic display. Thereby, information was obtained on the asynchronous cell population prior to its injection into the Sanderson chamber for the elutriation process. Profiles of cell population distribution were similarly obtained for all fractions collected during the elutriation. The G1 population was selected on the basis of narrowest size distribution around a mean 9.5 μm diameter cells. The selected fraction of G1 cells was then diluted to $7x10^4$ per ml for convenience of use for the single cell gel (SCG) electrophoresis preparation following X-irradiation.

SYNCHRONOUS POPULATIONS

Since the S/S cells grow in suspension, the synchrony method developed by Nagasawa *et al.* (1980) utilised centrifugation through a continuous 2-10% gradient of sucrose in full culture medium. A synchrony method using a specially modified Beckman JE6 Centrifugal Elutriation system was developed by Keng *et al.* (1981); the elutriation method enabled investigators to obtain larger and more pure populations of cells for study than did the continuous sucrose gradient method. In addition, the sucrose gradient method requires the use of tritium suicide to remove contaminating S-phase cells from the population, but the elutriation method eliminates the need for radioisotopes. The technique of centrifugal elutriation, rather than chemical or thermal methods, was used to synchronise the L5178Y S/S cells, to avoid interference with metabolic processes and cell cycle kinetics.

Once cell populations were separated by elutriation, cell volumes and concentrations were confirmed immediately using a Coulter Channelyzer system. Later, independent confirmation of the synchrony, based on relative DNA content was obtained by cell flow cytometry of the various fractions. After the desired population of cells in G1 was accumulated, cells were randomised into separate exposure vessels and exposed to X-irradiation at a density of $1x10^5$ cells /ml. In normal growth the G1 phase lasts for ~three hours based on a doubling time for asynchronous populations of 11 ± 1 hours (ideal cell density ranges from $1x10^4$ to $5x10^5$ cells/ml). Since the absence of DNA repair in G1 lasts for 0-3 hours post synchrony, that is ample time for transfer of cells between treatment venues, as long as temperature and pH are maintained (as shown by Nagasawa *et al.* 1980 and by Lett *et al.* 1989).

CELL ELUTRIATION

The Principle

Centrifugal elutriation allows the isolation of living cells at concentrations of up to 10^8 cells per ml without exposure to toxic media (eg tritium suicide alternative techniques). A specially modified Beckman J2-21 centrifuge was used with a Beckman JE-6B elutriator rotor (Figure 1). The JE-6B rotor separates particles according to their rate of sedimentation by centrifugal elutriation; the tendency of cells/particles to sediment in a centrifugal field is offset by a liquid flowing in the centripetal direction. When the centrifugal and counter-flow forces are balanced, a range of particle sizes can be kept in suspension. When the flow velocity is increased, or the *g*-forces are reduced, particles with low sedimentation rates are washed (elutriated) out of the rotor, leaving particles with higher sedimentation rates remaining in the rotor (Figure 2). Thus, lighter particles are flushed from the rotor by incrementally either increasing the flow rate or reducing the rotor speed. This phenomenon is exploited in the separation of cells according to their stage in the cell cycle as a function of cell size (i.e., cell size/volume increases as cells progress through the cell cycle); for a given cell concentration cells are smaller in G1 than in later stages (S and G2) prior to mitosis. Using this principle, cells in different stages of the cell cycle can be separated.

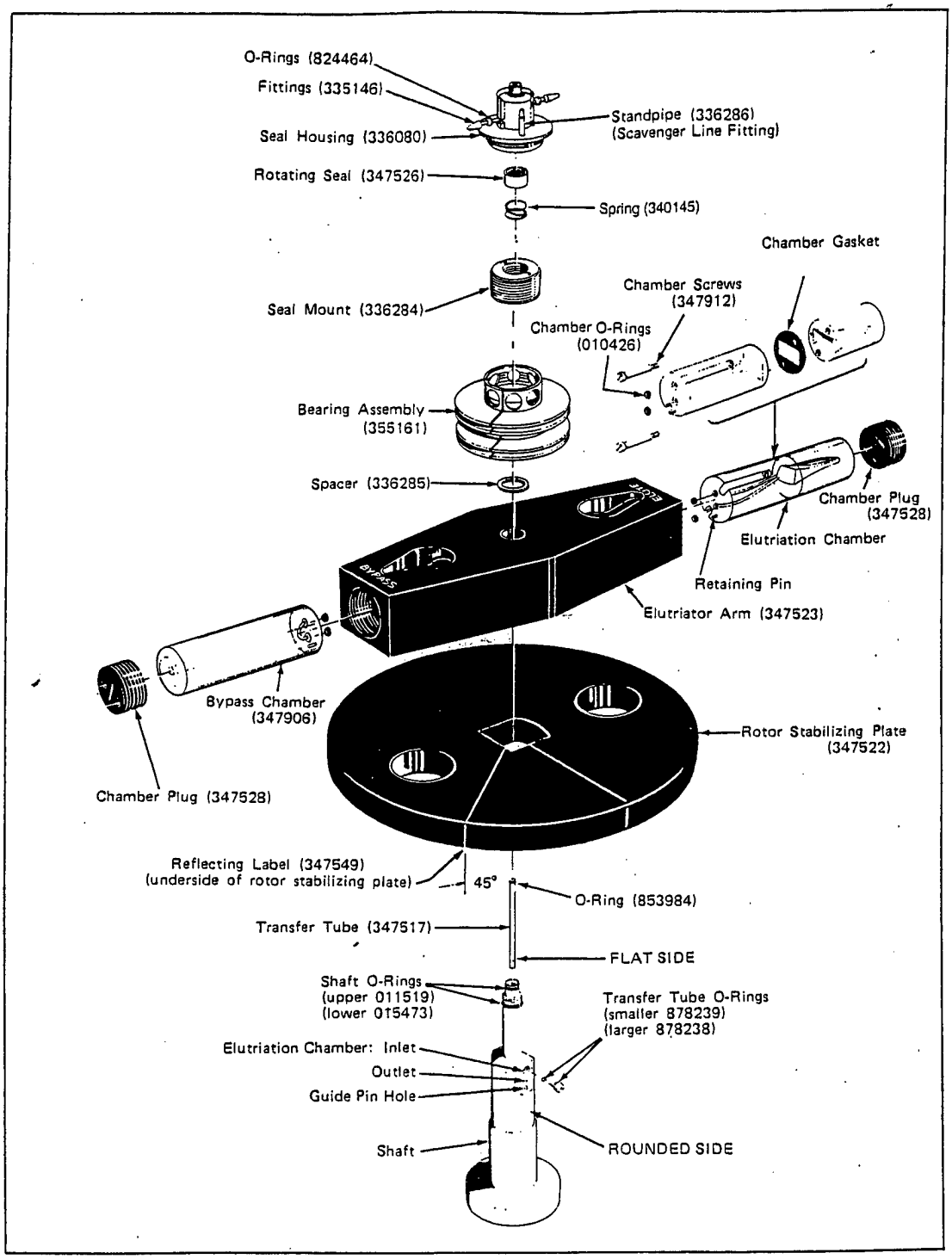


Figure 1. Exploded view of the assembly of a Beckman JE-6EB rotor and elutriation chamber. A cell suspension (~5x10^7/ml) is injected in a volume of 20 mls into the chamber while the rotor spins at 2550 rpm. Details in Appendix 4.

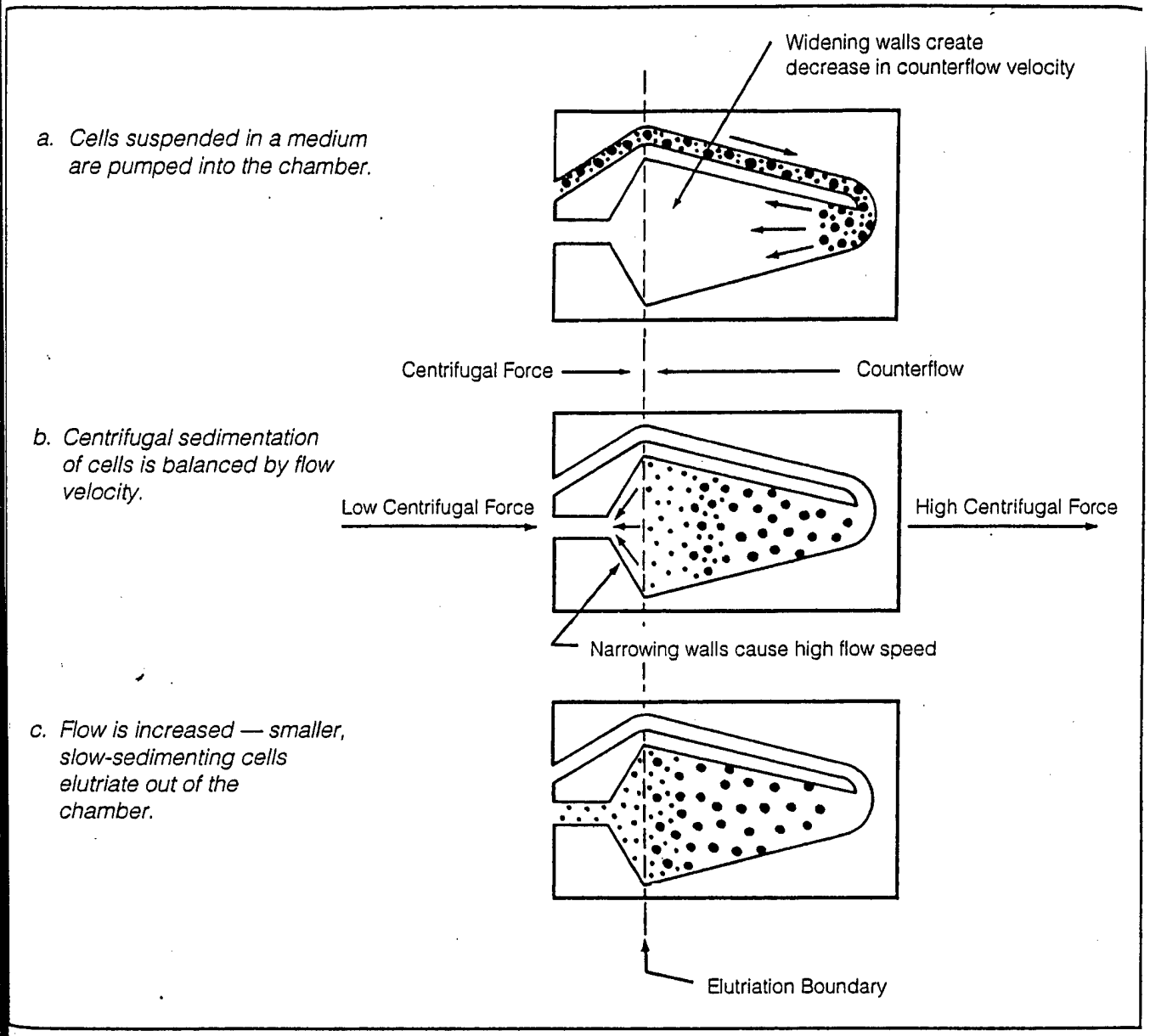


Figure 2. Schematic of elutriation chamber within the Beckman centrifuge JE-6B rotor showing cell flow, collection and elutriation with changes in centrifugal versus centripetal force. In our studies flow rate was maintained constant at 20 ml/min while rotor speed was reduced.

A nomogram (Figure 3) used to select optimum rotor speed and fluid flow velocity was generated from the equation,

$$F = XD^2\left(\frac{rpm}{1000}\right)^2$$

where, F = flow rate in ml/min

X = a constant, 0.0378 for the Sanderson chamber

D = particle diameter in μm

Rpm = rotor speed in revolutions per min.

The equation is an expression of conditions at the elutriation boundary, derived by setting the velocity of a particle sedimenting in a gravitational force field (Stoke's Law) equal to the flow velocity at the elutriation boundary. Flow velocity is equal to the flow rate (ml/min) divided by cross-sectional area (cm^2). In the equation, X is a constant that incorporates the cross-sectional area of the chamber at the elutriation boundary, the radius at the elutriation boundary, the density difference ($\Delta\rho = 0.05\ g/ml$ for biological cells), the viscocity of the medium ($\eta = 1.002$ mPa.s) and factors that convert rpm to radians per sec.

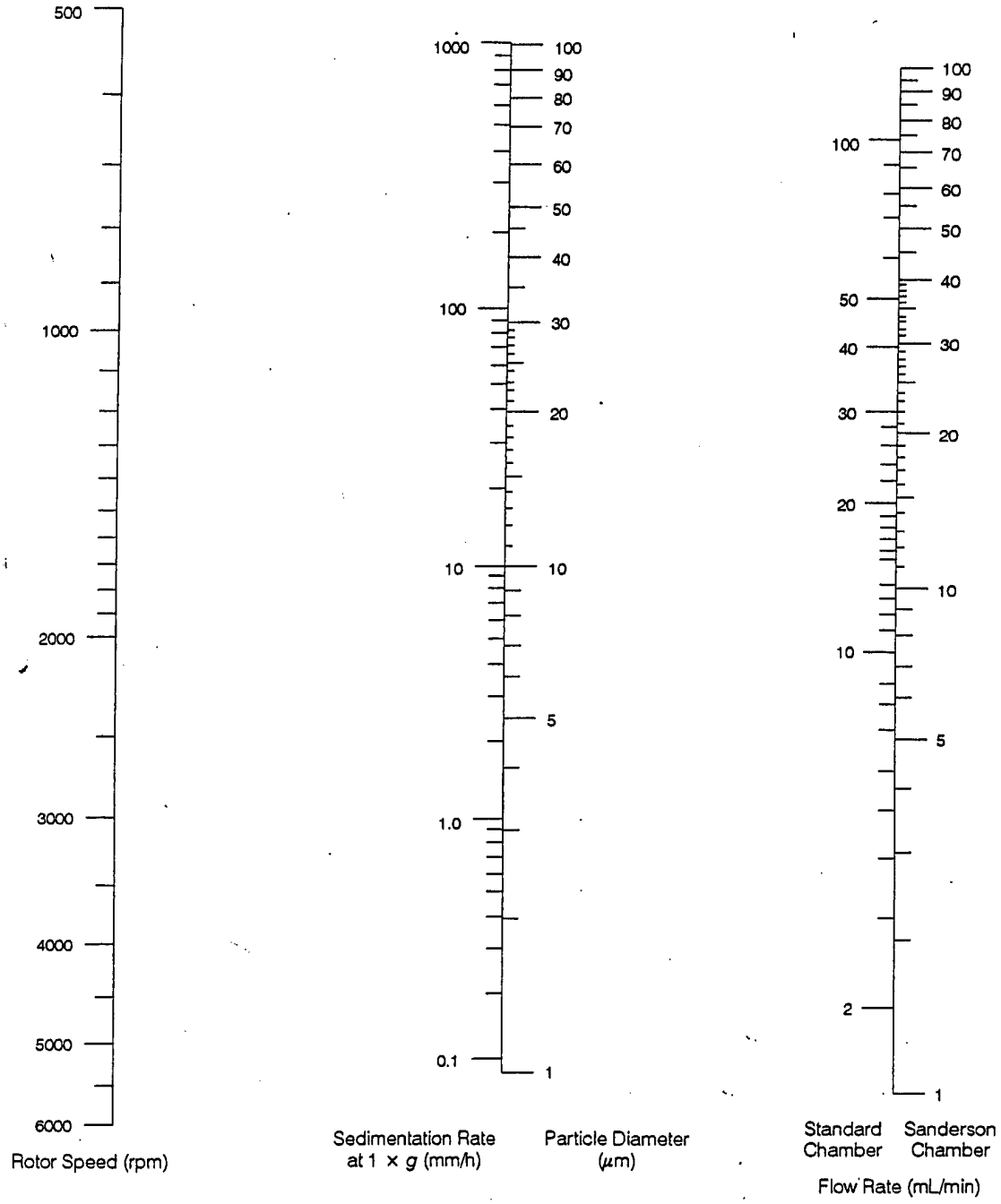


Figure 3. Nomogram used as a guide to select the combination of flow velocity and centrifuge rotor speed to collect L5178Y S/S cells of 9.5 μm diameter for G1.

The Technique

The rotor of the Beckman centrifuge contains a 5.9 ml capacity Sanderson chamber into which a sample of cell suspension containing approximately $1x10^8$ cells was introduced through the narrow centrifugal end at the preset flow velocity while the rotor was spinning at a pre-determined constant velocity. The flowing liquid, cells suspended in culture medium, sweeps cells with low sedimentation rates towards the centripetal area of the chamber. The cell sample in the Sanderson chamber was viewed, through a port in the lid of the centrifuge, by strobe light synchronised with the rotor speed.

The Beckman J2-21 centrifuge was modified specifically for this purpose. Care was taken to avoid unnecessary vibrations during the process. A harness was attached to the upper bearing assembly/rotating seal to prevent undue tension on the inlet and fraction collection tubing during acceleration or deceleration of the rotor. The centrifuge vacuum pump was inactivated and the rotor temperature control was set at 30°C to avoid activating the refrigeration unit. This was particularly important because, when activated, the temperature plunges rapidly to levels approaching 10°C. The L5178Y S/S cells are intolerant of such low temperature. The elutriation procedure was operated at room temperature although the rotor temperature increased to approximately 28°C by the completion of an elutriation. Fine control of rotor speed was achieved by switching an over-ride of the coarse adjustment (its standard rotary knob is turned fully anti-clockwise) to use a potentiometer with rotary switch and Vernier graduations. The centrifuge was also fitted with an additional digital display of rotor speed. A photograph of the apparatus is shown in Figure 4. The rotor speed was calibrated against the speed control Vernier settings on the Beckman J2-21 centrifuge.

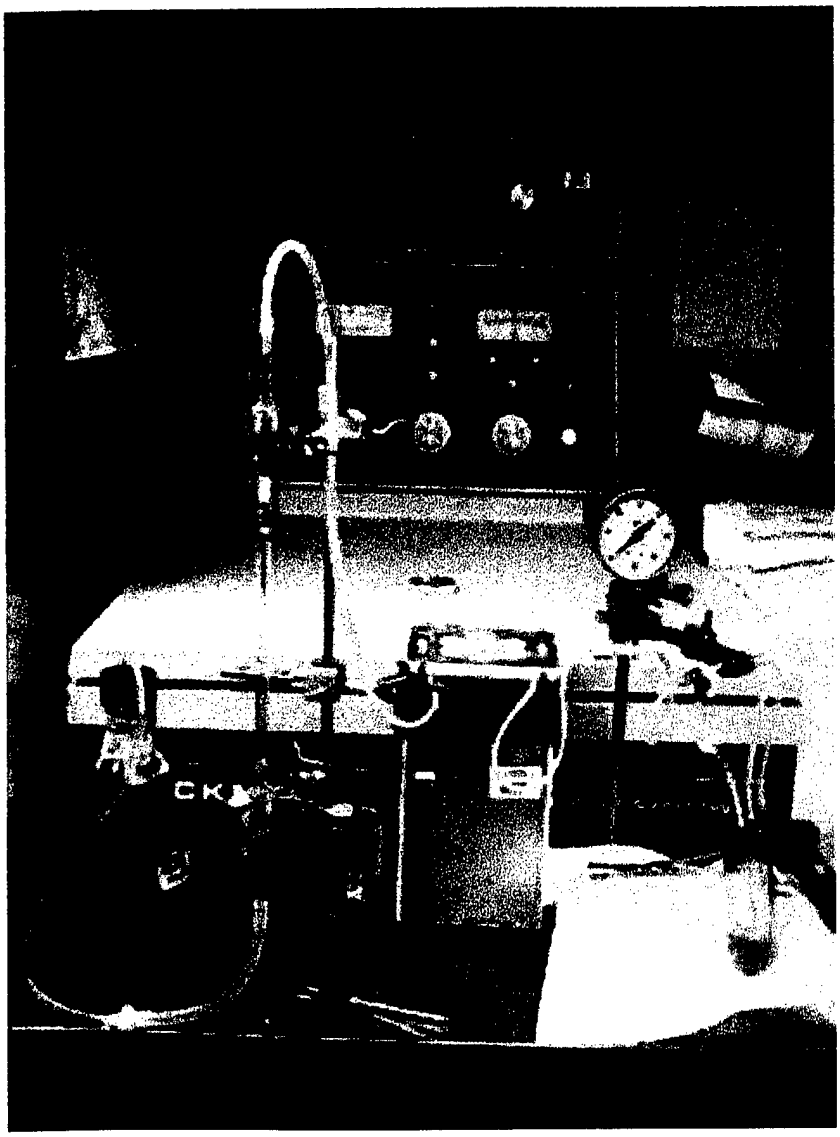


Figure 4. Photograph of apparatus used for elutriation. The large conical flask on the left of figure provides the supply of growth medium that is pumped through the system at 20 ml/min. A peristaltic pump provided constant velocity of flow and the rate in the system was calibrated against a manometer. Pressure was monitored by the in-line pressure gauge as an indicator of bubbles in the rotor. To the right of the figure is the injection vessel into which 20 ml cell suspension in introduced. (Details of the sequence are given in Appendix 4)

Calibration of Beckman centrifuge rotor speed against fine Vernier Settings: June 1999 and original data

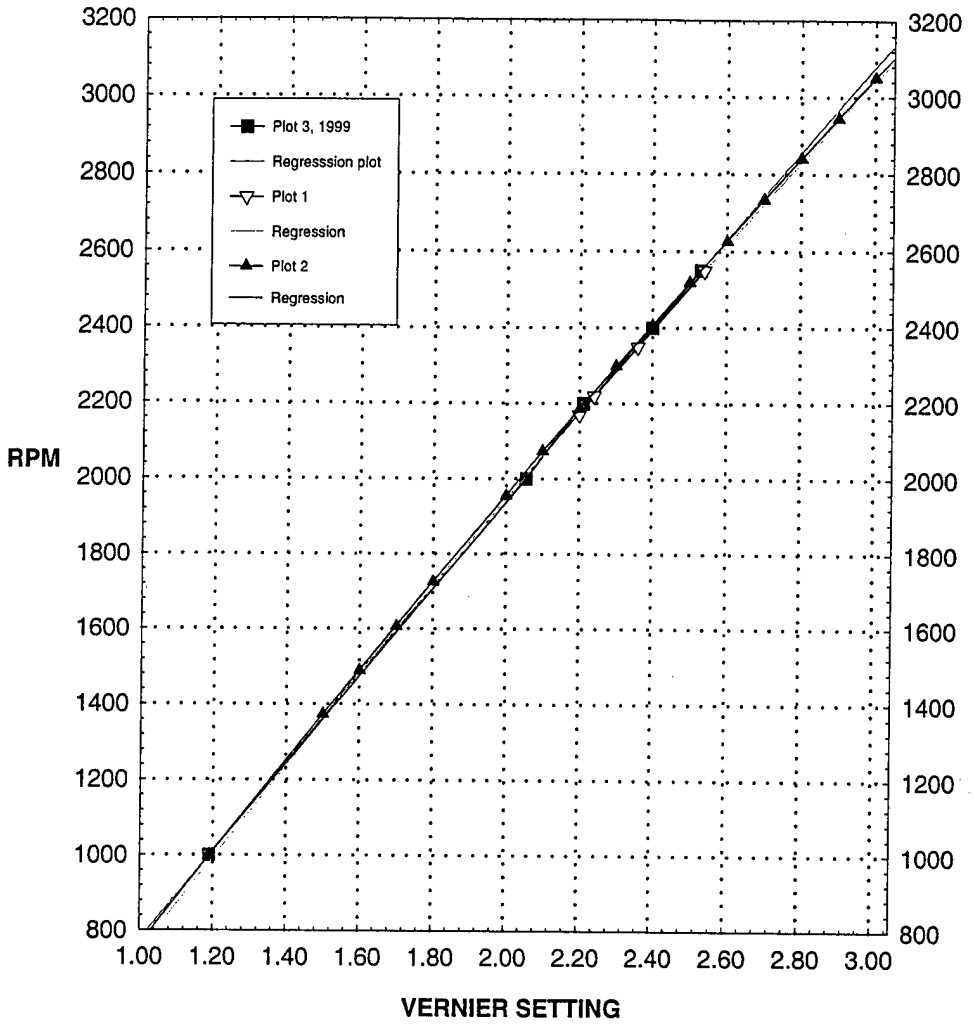


Figure 5. Calibration graph for settings of the fine vernier control against the digital display of speed on the modified Beckman J2-21 centrifuge.

The flow system consists of a pressure gauge for monitoring back pressure in the system caused by air bubbles in the rotor, a polycarbonate bottle for storing the cell culture sample prior to delivery into the rotor, silicon medical tubing and three-way valves to route the liquid and cell suspension to and from the rotor. A variable speed peristaltic pump (LKB 2115) provided the driving force and the velocity of fluid flow in the system was calibrated with a flowmeter (Manostat, NY). Settings of the three-way valves used in the procedure are shown schematically in Appendix 4. The velocity of flow of medium through the Sanderson chamber was measured and used to calibrate flowmeter readings against the Vernier graduation on the LKB peristaltic pump (Figures 5 and 6).

Throughout the elutriation procedure the in-line pressure gauge was used to constantly monitor back-pressure from the rotor. This was particularly important during injection of the cell sample into the rotor.

Cells in G1 were separated out of an asynchronous population of L5178Y S/S cells while still suspended in culture medium. These cells normally grow in suspension and they tolerate the elutriation process without adverse consequences provided that they do not remain at suboptimal growth temperature for extended periods. The separation process was undertaken at room temperature. The combination of rotor speed and pump velocity required to provide optimum separation was determined from the nomogram (Figure 3) after calibration of the centrifuge rotor velocity and the peristaltic pump. The yield of cells synchronised in G1 are then available for use in experiments. We chose to maintain a constant velocity of 20 ml/min flow of medium whilst carefully reducing the rotor speed through predetermined increments.

Although we were primarily interested in collecting samples with G1 cells, other size fractions were also collected for comparative measurement of DNA content using CFC and comet assay techniques. A 20 ml volume asynchronous source sample containing approx. $2x10^8$ cells was injected with the rotor speed operating at 2550 rpm. All cell fractions were collected in volumes of 50 ml in 150 seconds. One fraction was collected during injection and three more fractions were taken without changing the rotor speed.

As soon as the last fraction was collected at 2550 rpm, the rotor speed was slowly decelerated to 2350 rpm using the fine Vernier-graduated control. Rapid change in speed setting causes fast deceleration and invokes the rotor brake to temporarily reduce speed too far. This can result in mixing of the cell fractions. It may take 20 – 30 sec to carefully change the rotor speed, but it was considered to be essential for good sample separation. Four fractions were collected at 2350 rpm.

After the last fraction was collected at 2350 rpm the rotor speed was reduced to 2220 rpm for another four 50 ml fractions. As each fraction was collected the size distributions and numbers of cells was measured using the Coulter counter and channeliser. This allowed the opportunity to make small changes in rotor speed, if required. We anticipated the best separation of G1 populations during elutriations at the rotor speed of 2220 rpm. For comparison of data from larger cells (later in the cell cycle) larger adjustments in rotor speed were used and fractions were collected at 1980 and 1750 rpm. After the desired populations were collected the remaining cells were flushed out of the rotor by turning off the rotor drive and allowing the rotor to come to a complete stop. These cells were also collected and their size distribution measured. (For detailed description of the elutriation protocol refer to Appendix 4)

The rotor was cleansed of culture media and cells by washing with approximately 500 ml distilled water with the rotor running at 1000 rpm. This ensures that bubbles do not collect in the rotor. In order to adequately flush the system the rotor must be operating. Finally, the rotor was removed, dismantled, washed with deionized water and air dried. All jets and "O" ring seals were carefully inspected for possible leaks before re-assembling the rotor.

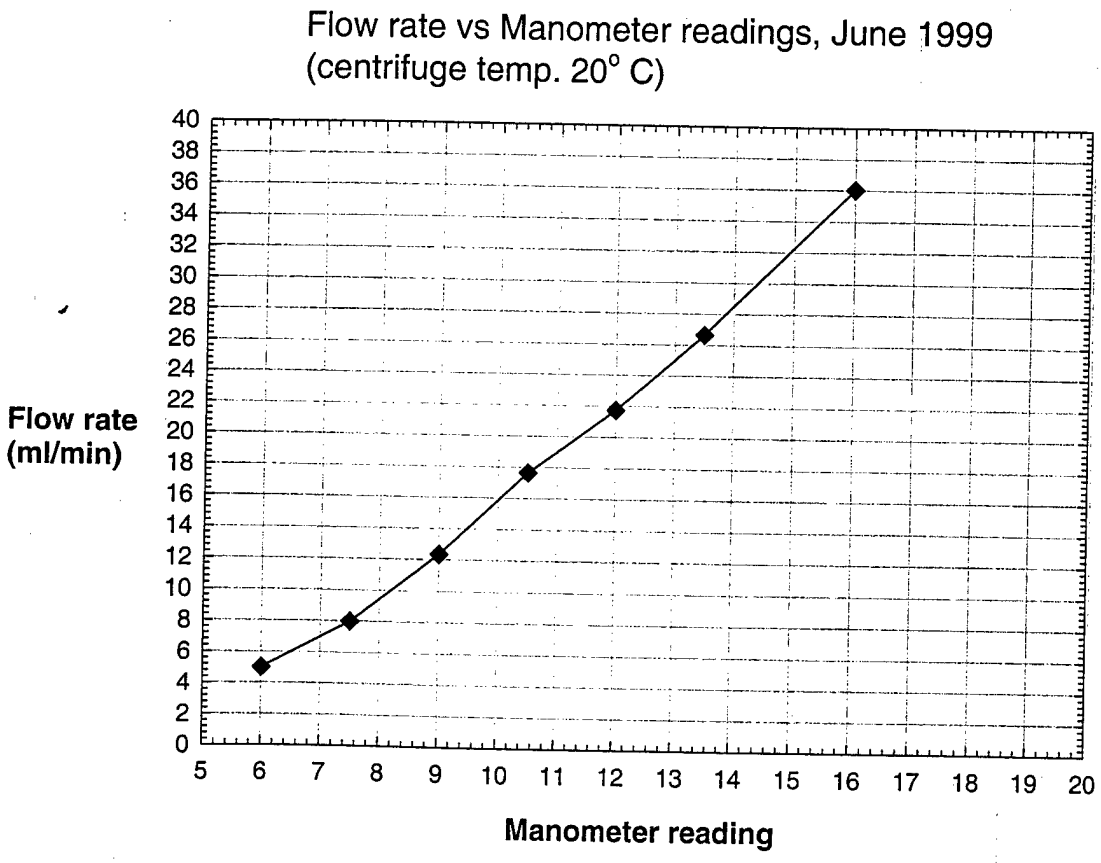


Figure 6. Calibration curve for liquid flow rate (20°C) through the Sanderson chamber at rotor speed 2550 rpm. for a fixed setting (Vernier graduation 5.07) on LKB peristaltic pump. (Flowmeter - Manostat, NY).

COMET ASSAY

The Principle

The comet assay, or single cell gel (SCG) or microgel electrophoresis (MGE) assay allows direct visualization of DNA damage in individual cells (Fairbairn *et al.* 1995). In this procedure a small number of irradiated cells is suspended in a thin layer of agarose gel on a microscope slide, lysed in alkali solution and exposed to an electric field. The charged DNA is pulled from the cell nucleus and the fragments are distributed in the form of a tail in the so-called "comet". The SCG electrophoresis process allows examination of DNA breaks in individual cells. Briefly, a cell suspension of approx. $8x10^4$ cells per ml is diluted into agarose so that approximately 20,000 cells are spread onto each microscope slide. The cells are then subjected to alkaline hydrolysis in which binding protein is digested away from DNA and a denaturing alkaline solution is used to reduce the DNA to single strand fragments which are then free to migrate in the electric field in an electrophoresis bath. The sugar-phosphate backbone gives the DNA a strong negative charge so that fragments will migrate towards the anode. The charge-to-size ratio for DNA is relatively constant, however, the agarose matrix restricts migration making it more difficult for the larger fragments. Because it is easier for smaller fragments to weave through the matrix, the DNA fragments separate according to size and produce the characteristic "comet tail" of DNA drawn out of the nucleus.

Original experiments with ionizing radiation (Ostling and Johanson 1984) showed that the extent of liberated DNA fragments, i.e., the length of the tail, is related to the dose of X-irradiation. Lai and Singh (1995, 1996) used a modified technique which simply measured the length of the comet tail and compared results for controls and RF irradiated cells.

This approach does not consider the amount of breakage, but simply shows the greatest distance of travel of DNA fragments. Hence, a very small single fragment would travel further than a larger number of bigger fragments. A more appropriate method may be to quantify the "tail moment", defined as the product of the tail length and the % of total DNA in the tail. Tail length is defined as the distance in the X-direction from the centre of gravity of the head to the centre of gravity of the tail. Thus, tail moment incorporates a measure of both the smallest detectable size of migrating DNA and the number of broken pieces.

The values obtained for the comet assay differ quite markedly according to the type of measurement. Bocker *et al.* (1997) showed that measures obtained, in lymphocytes, for tail moment and fluorescence ratio are similar and show a consistent dose-response to X-irradiation.

The Technique

The comet assay experimental protocol is outlined in Appendix 2. Microscopic fluorescent images of a comet from each cell are digitised and stored on computer. A typical comet image is shown in Figure 7. The use of computerized image analysis systems and custom-written software allows the total comet fluorescence to also be determined as a function of DNA content (Olive *et al.* 1992). Expertise in the SCG technique was developed by the group at Colorado State University (CSU,VTH) under guidance of Dr. Olive, a pioneer of the assay.

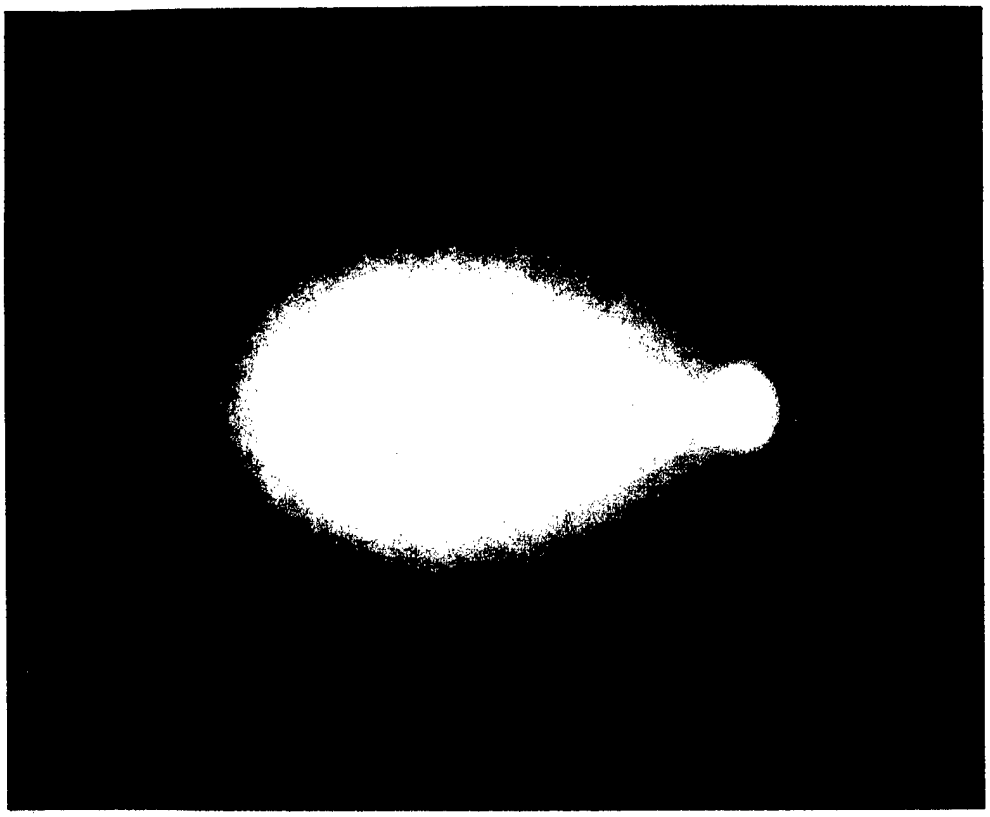


Figure 7. Image of a "comet" of DNA from a single cell obtained after hydrolysis and electrophoresis.

The image analysis process begins with selection of an individual microscopic image of a cell; this being labour-intensive and subjective and therefore subject to error. The next stage of the process is the image segmentation, or computer-capture, and this is followed by feature identification (computer acquired, subject to variations in fluorescence relative to background noise and detection of edge effects). The present study used the facilities at the CSU and used the measurement of comet tail length and comet tail moment as measures of single strand DNA damage. This system does not appear to include compensation for changes in grey level intensity or image threshold. However, to maintain relativity the camera setting was fixed at the beginning of the image analysis procedure and remained constant throughout each experiment. Microscopic fluorescence images of DNA comets were digitized and stored in MS Excel spreadsheets. Analysis and graphical representations of data were undertaken using Sigma-Plot software.

In order to identify relatively small changes in single strand breaks, it is essential to carefully control all conditions of cell culture and DNA hydrolysis. From private communications on his technique, Singh emphasises the importance of; (a) using proteinase-K to remove proteins binding DNA molecules and to allow the single strands to unravel, (b) completing all single cell gel procedures in low-level lighting, preferably gold, to avoid photolysis and enhancement of ssb, (c) undertaking all procedures on ice to reduce enzyme activity/repair during SCG process, (d) circulating solutions, particularly during electrophoresis when necessary to ensure complete removal of salts in the gels.

CELL FLOW CYTOMETRY

During the elutriation process samples of $2x10^6$ cells were collected from each fraction and fixed in ethanol/citric acid buffer for analysis by cell flow cytometry (CFC) for confirmation of DNA distribution (see Appendix 1 for details of preparation). Fixed cells were kept in air-tight containers in a cold room at 4°C until DNA analysis was undertaken. Cells were normally fixed immediately after fraction separation for subsequent analysis of DNA content by cell flow cytometry. In some samples there was a deliberate delay of 3-4 hrs before fixation to ascertain the potential effects of apoptosis on the G1 fraction of these L5178Y S/S cells.

IONIZING RADIATION

The single cell gel electrophoresis technique was used with the Comet assay to determine the presence of single strand breaks in DNA following exposure to X-ray at a range of doses; 0.0, 0.5, 1.0, 2.0, 3.0, and 4.0 Gy.

The 50 ml elutriation sample of G1 cells was diluted to a concentration of $5x10^4$ per ml in phosphate buffered saline, sheilded from direct light and taken immediately to the X-ray facility.

Lymphocytes from fresh whole blood were similarly irradiated and used as a control (see Appendix 3 for details of the preparation). The state of the art X-irradiation facility at the Veterinary Teaching Hospital, Colorado State University was used for this purpose under the authority of Prof. Sue LaRue. The X-ray facility and exposure condition is shown in the photograph (Figure 8).

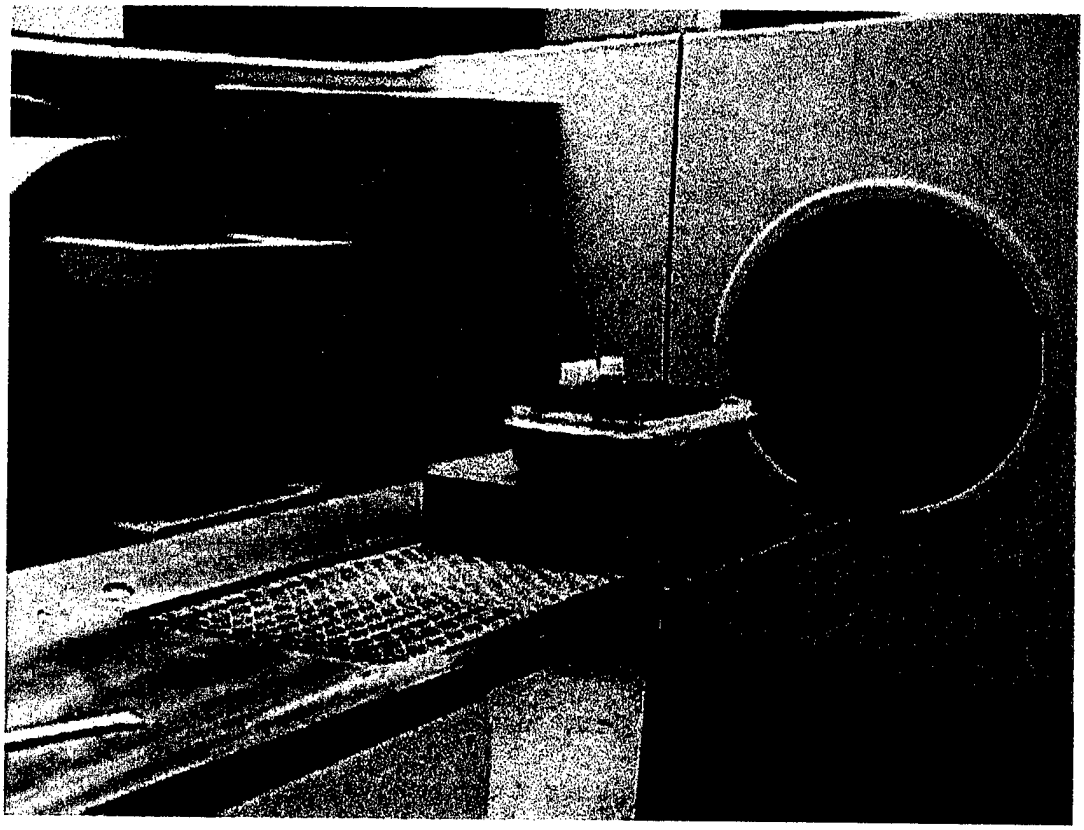


Figure 8. Photograph of the X-ray source used to irradiate cell samples.

RESULTS

CELL SYNCHRONISATION

Figure 9 shows plots of cell size distributions from typical results of centrifugal elutriation used to separate populations of G1 cells from others. Asynchronous population distributions are shown for comparison.

Based on cell size, the G1 fractions were obtained with a peak profile around 9.5 μm. The optimum yeild of G1 fraction was collected at a rotor speed of 2220 rpm although it was also possible to obtain reasonable yield at 2350 rpm. It is clear from the series of graphs shown in Figure 9 that the size of cells per sample increases as fractions are collected at decreasing rotor speeds. The lowest rotor speed used in our experiments did not yield significant numbers of G2/M cells; those cells were of no interest to our study and would be washed out in the final fractions when the rotor was stopped.

These data confirm the sensitivity of this technique and the consistency of data using the L5178Y S/S cell line.

Figure 9. Size distributions for populations of L5178Y S/S cells collected as fractions of the original asynchronous population as a function of rotor speed.

9A – asynchronous "injection" sample at 0 rpm,

9B – first collected fraction at 2550 rpm,

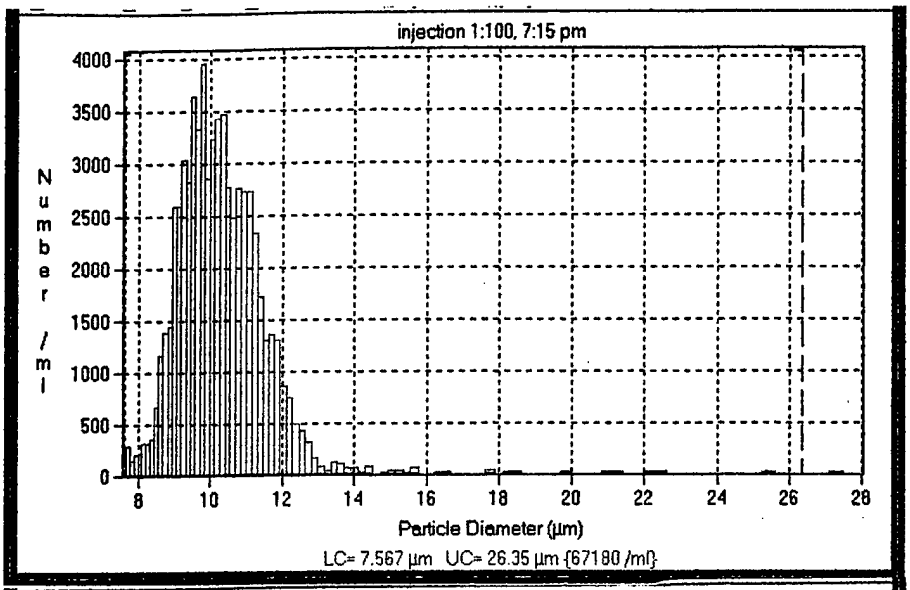
9C – fraction collected at 2350 rpm,

9D – fraction collected at 2220 rpm

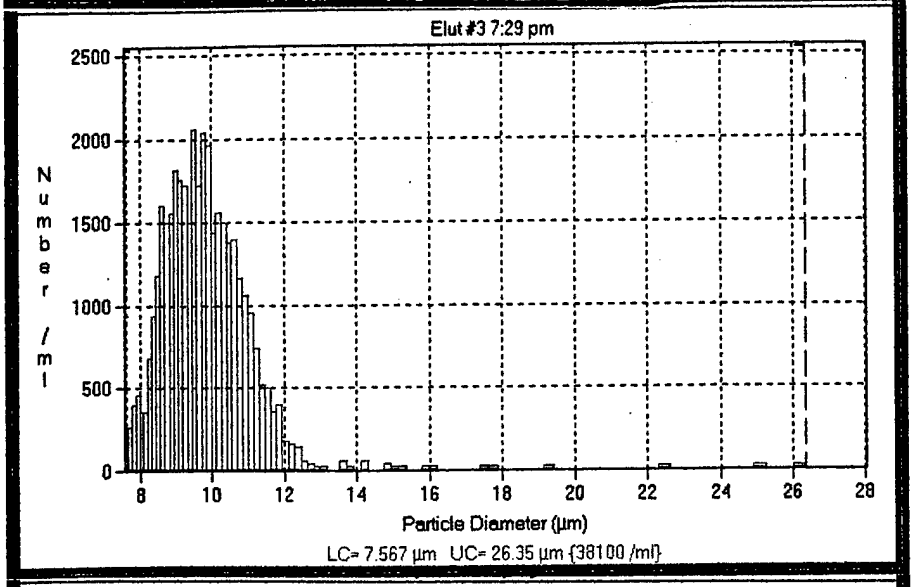
9E – fraction collected at 1970 rpm

9F – fraction collected at 1750 rpm

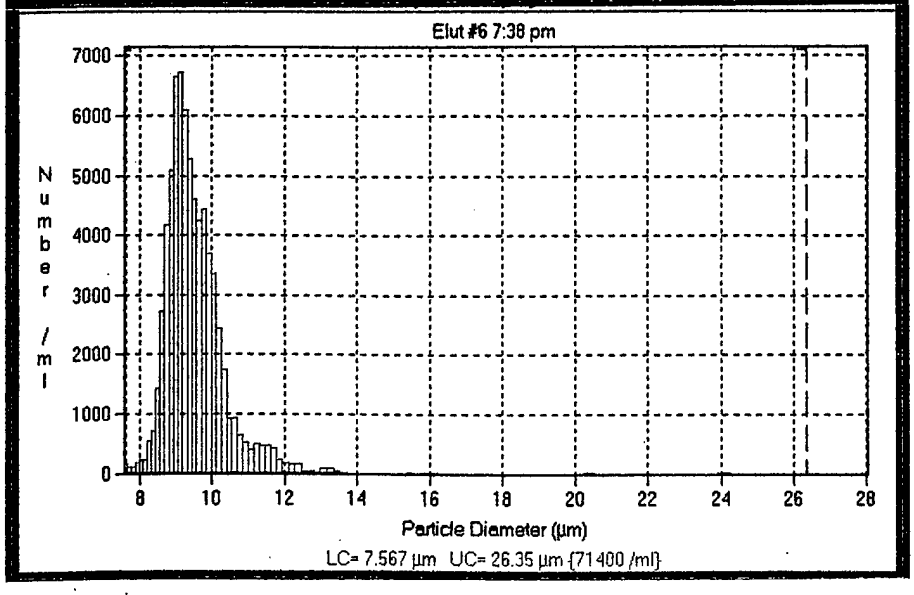
9A


injection 1:100, 7:15 pm
Number / ml
4000
3500
3000
2500
2000
1500
1000
500
0
8
10
12
14
16
18
20
22
24
26
28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {67180 /ml}

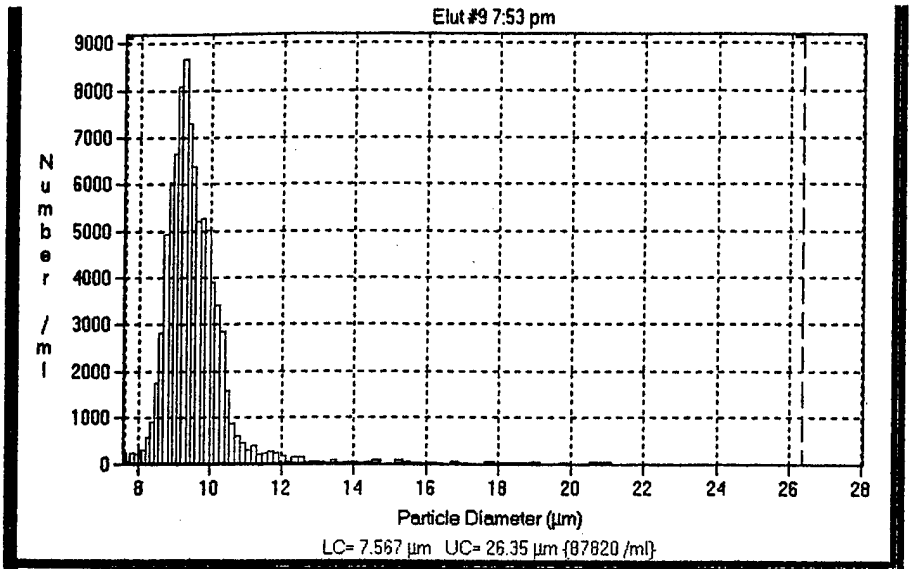
9B


Elut #3 7:29 pm
Number / ml
2500
2000
1500
1000
500
0
8
10
12
14
16
18
20
22
24
26
28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {38100 /ml}

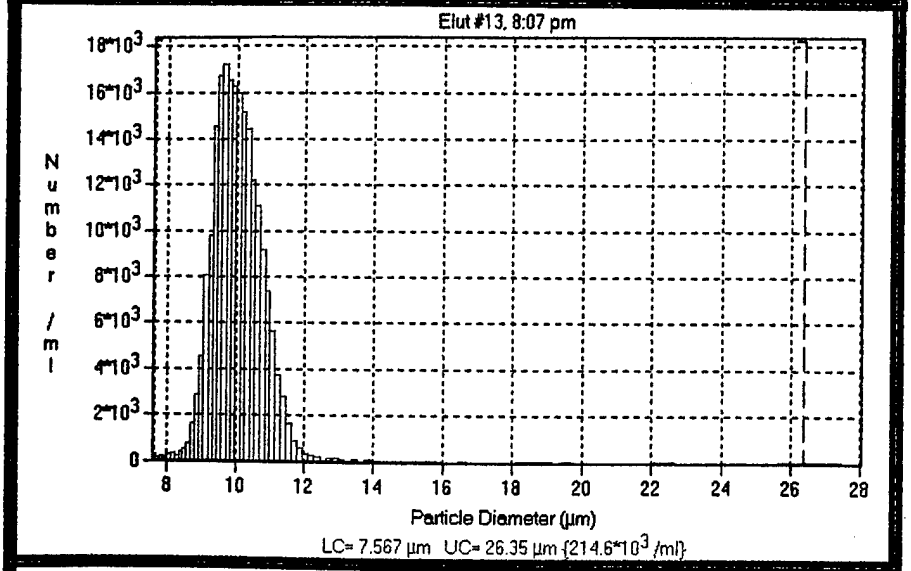
9C


Elut #6 7:38 pm
Number / ml
7000
6000
5000
4000
3000
2000
1000
0
8
10
12
14
16
18
20
22
24
26
28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {71400 /ml}

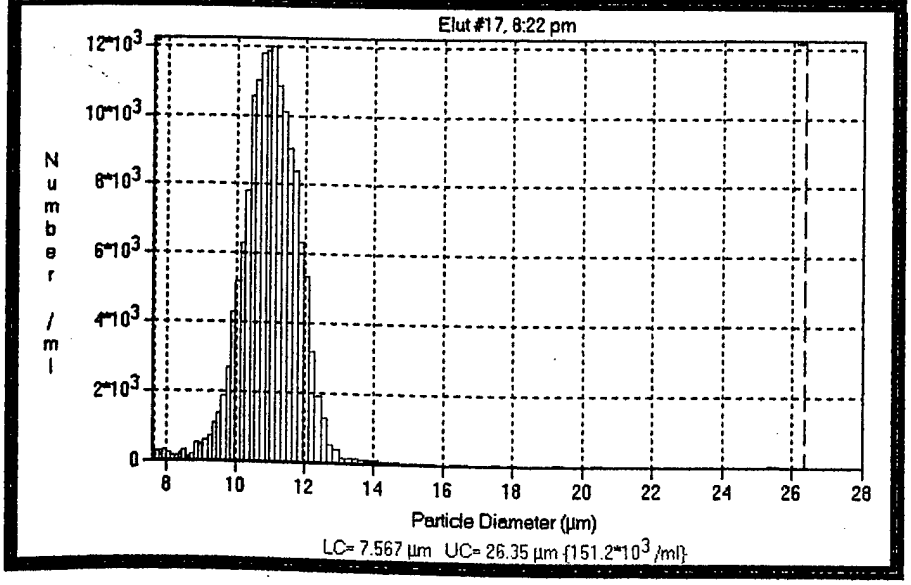
9D


Elut #9 7:53 pm
Number / ml
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
8 10 12 14 16 18 20 22 24 26 28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {87820 /ml}

9E


Elut #13, 8:07 pm
Number / ml
18*10^3
16*10^3
14*10^3
12*10^3
10*10^3
8*10^3
6*10^3
4*10^3
2*10^3
0
8 10 12 14 16 18 20 22 24 26 28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {214.6*10^3 /ml}

9F


Elut #17, 8:22 pm
Number / ml
12*10^3
10*10^3
8*10^3
6*10^3
4*10^3
2*10^3
0
8 10 12 14 16 18 20 22 24 26 28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {151.2*10^3 /ml}

% G1 Cells in Elutriation Fraction

The centrifugal elutriation process was able to consistently separate out fractions of cell populations in G1. The cell size distribution obtained from Coulter counter/channeliser procedures showed consistent narrow size distributions (see Figure 9) with ample quantities of G1 cells for our experimental purposes. The percent G1 cells within each synchronised fraction was calculated from cell flow cytometry (CFC) data and typically yielded populations of >90% for fractions obtained at a rotor speed of 2220 rpm.

Table 1 shows a clear correlation between collected cell sample size and centrifuge rotor speed. The data are representative, being obtained from a series of elutriation fractions from a single asynchronous source sample. Normally, for the purpose of the bioassay experiments, G1 fractions only would be collected and used for comet assay and CFC analysis. The highest yield of G1 cells measured by CFC was 99% for the 2220 rpm fraction.

Table 1. Centrifugal Elutriation showing representative data for % G1 and other fractions, calculated from relative DNA content in cell flow cytometry assays, as a function of altered rotor speed. G1 fractions for comet assay were obtained at 2220 rpm. Asynchronous source population is shown at 0 rpm. Corresponding DNA content profiles are given in Figure 11.

Rotor speed (rpm)	%G1	%S	%G2/M	Figure 11 CFC #
0	41	41	18	1
2550	66	29	5	2
2350	85	11	4	3
2220	92	7	1	4
1970	<50	>50	--	--
1750	5	90	5	--

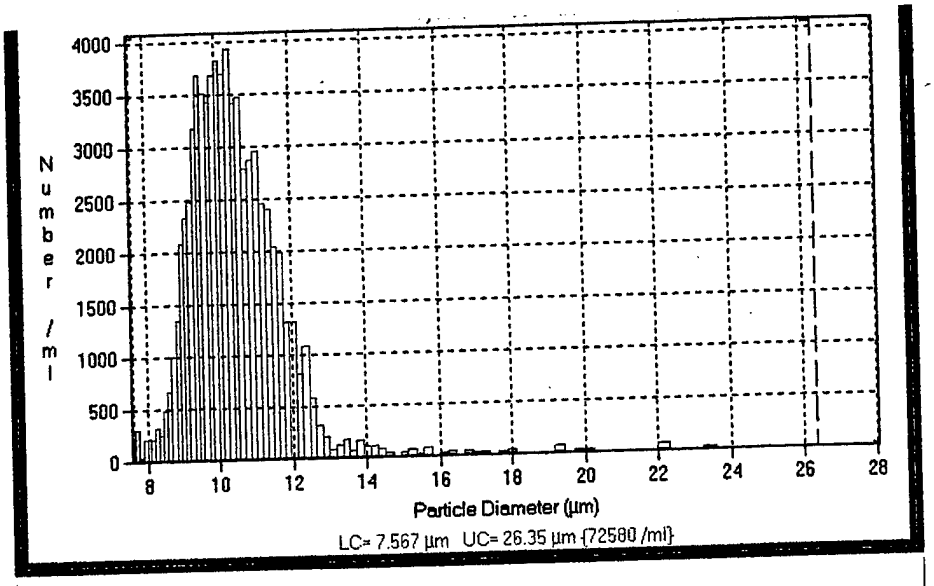
(-- = data not sufficiently accurate because computer analysis is normalized against G1 population which has been removed from slower fractions)

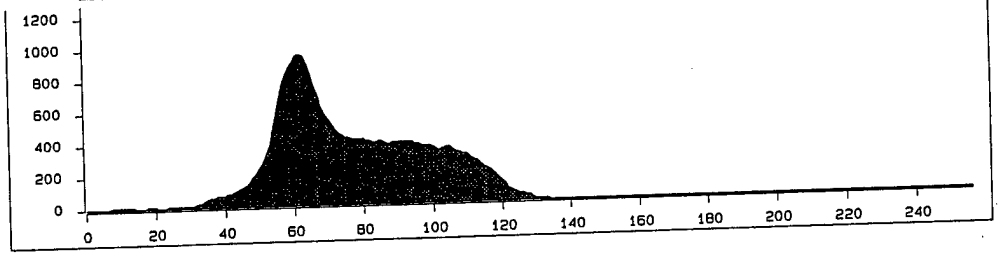
CELL FLOW CYTOMETRY

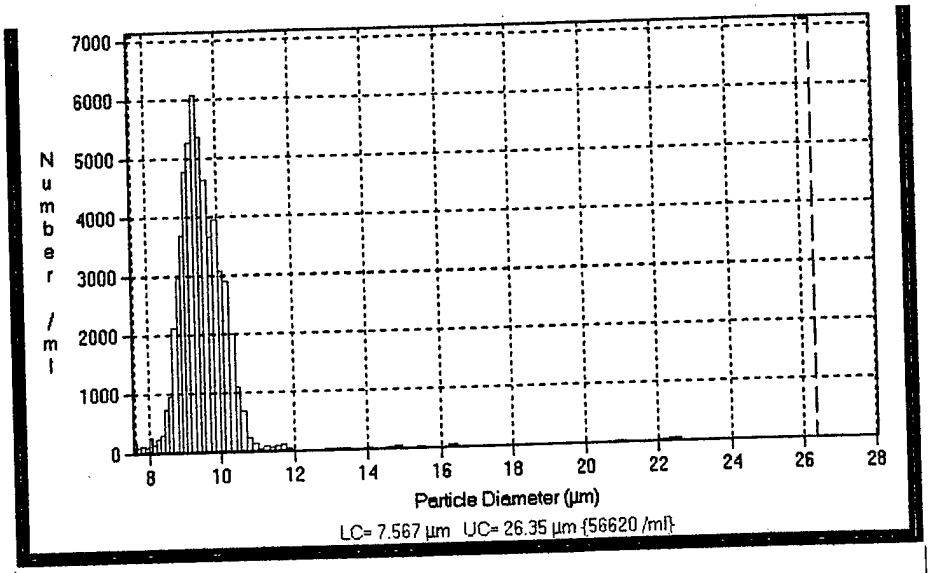
The data from cell flow cytometry studies show values for DNA content which are consistent with the cell size distribution profiles obtained for each of the elutriation fractions. Figure 10 shows that the CFC data confirm successful separation of cell fractions by size using the centrifugal elutriation technique. The profiles for DNA content shown in Figure 11 correspond to the actual elutriation fractions given in Table 1.

The importance of fully understanding cell cycle kinetics was demonstrated when delayed use of synchronised preparations was shown to directly affect the stability of the G1 fractions of L5178Y S/S cells. The CFC profiles showed evidence of apoptosis when isolated G1 cells were held at room temperature for three hours before fixation. The DNA content for apoptotic cells appears as a small blip on the curve before the G1 population (see Figure 12). Hence the comet assay should be able to distinguish obviously apoptotic data on the basis of the unusually large tail/head ratio.

Figure 10. Comparison of profiles for population cell size distribution (Coulter) and DNA content (Cell Flow Cytometry); asynchronous source cells (upper) and synchronous G1 elutriated cell population (lower). Note the similar tight distribution for cell size and DNA content in the G1 population of L5178Y S/S cells. (Yield of G1 fraction =96%)


Number / ml
4000
3500
3000
2500
2000
1500
1000
500
0
8
10
12
14
16
18
20
22
24
26
28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {72580 /ml}


1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
180
200
220
240


Number / ml
7000
6000
5000
4000
3000
2000
1000
0
8
10
12
14
16
18
20
22
24
26
28
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm {56620 /ml}

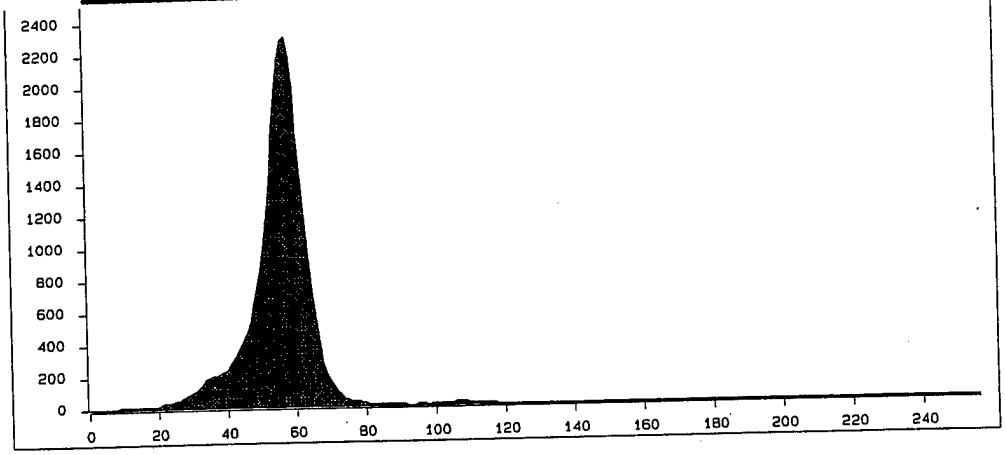

2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
180
200
220
240

Figure 11. CFC analysis of DNA content for L5178Y S/S cells; elutriated fractions corresponding to # 1-4 in Table 1.

- **Upper profile – asynchronous source population,**

  Bottom profile –92% G1 fraction

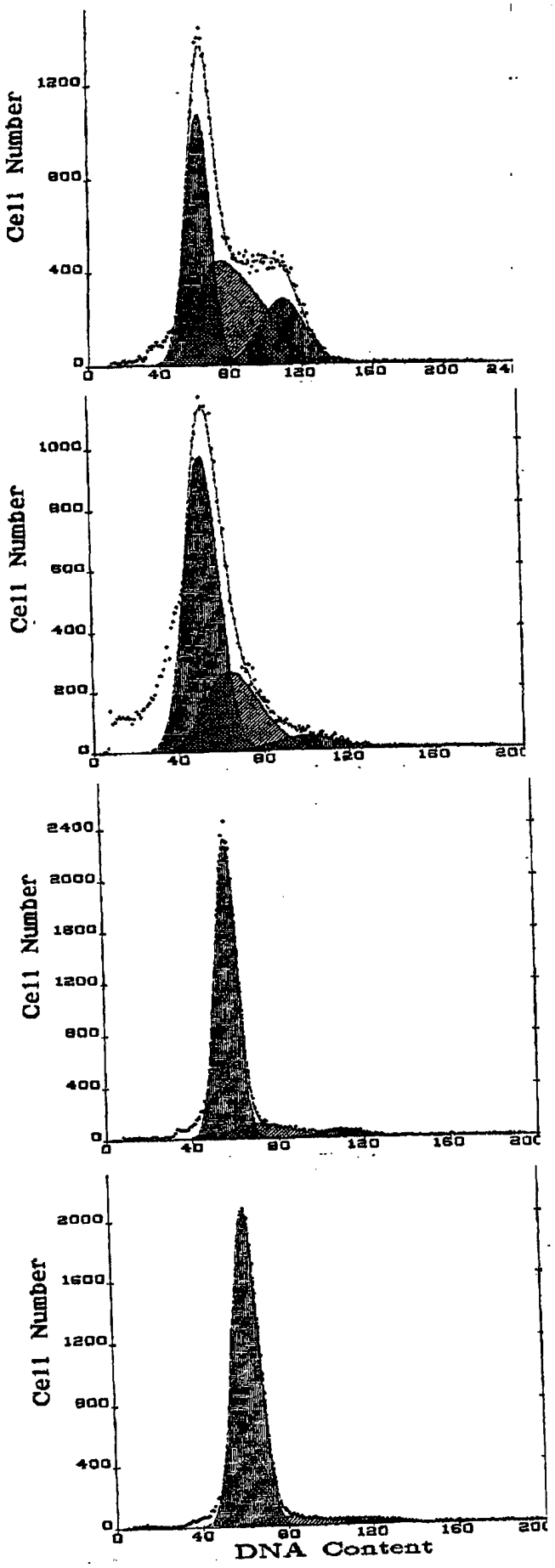
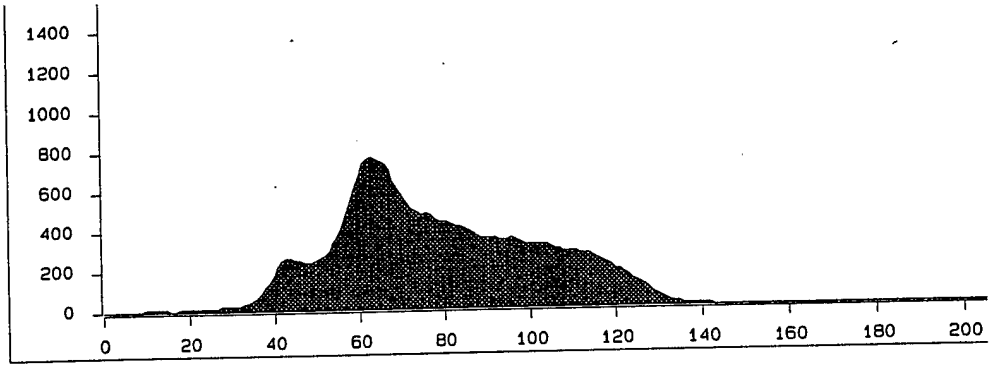
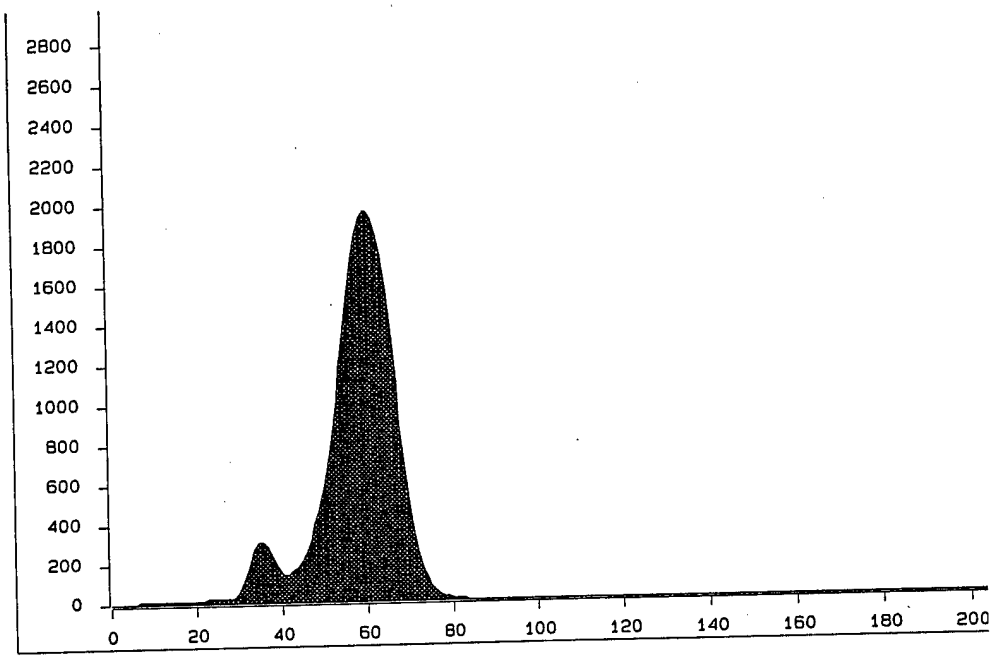
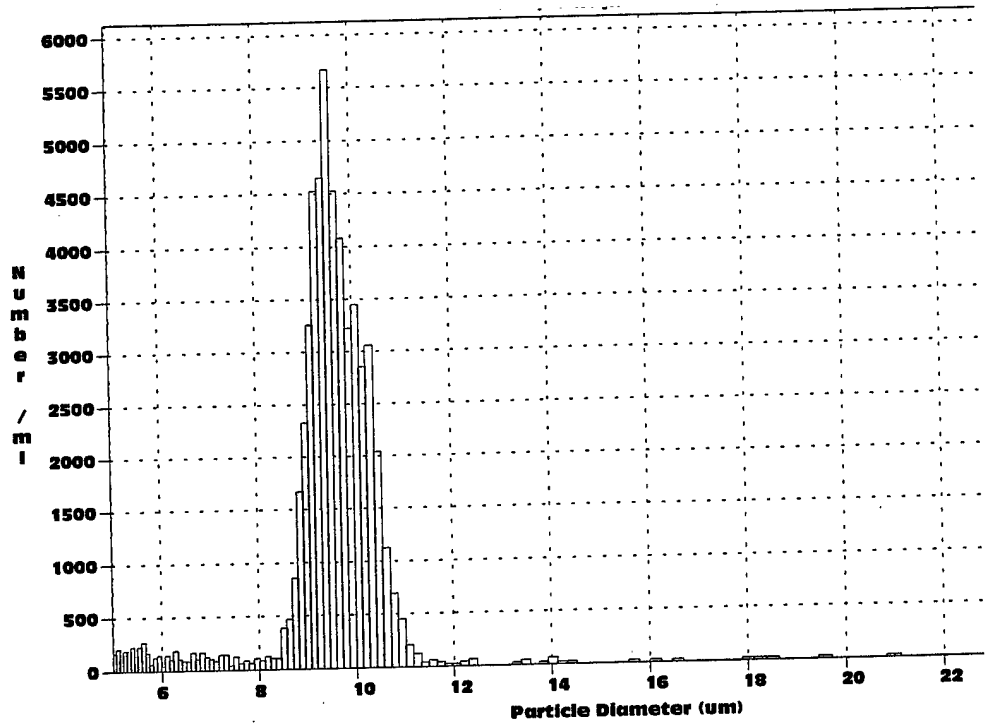

Cell Number
1200
800
400
0
0
40
80
120
160
200
Cell Number
1000
800
600
400
200
0
40
80
120
160
Cell Number
2400
2000
1800
1200
800
400
0
40
80
120
160
Cell Number
2000
1600
1200
800
400
0
40
80
120
160
DNA Content

Figure 12. Cell Flow Cytometry DNA content profiles showing aberrant distribution caused by apoptosis. Both asynchronous source and synchronous G1 samples of L5178Y S/S cells were held at room temperature for 3 hours after collection prior to fixation for CFC. (Sample size; $2.5x10^6$ cells)

The lower (Coulter) graph shows the size distribution in the G1 fraction measured immediately after collection by elutriation.


1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
180
200


2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
180
200


6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Number / ml
6
8
10
12
14
16
18
20
22
Particle Diameter (um)

COMET ASSAY

Tail moment

Results of exposure to X-irradiation over a range of 0–4 Gy show a weak dose response where the peak value can be seen to shift to the right, i.e. progressively increasing high value (see Figure 13). The tail moment data appeared to be the most consistent of the comet parameters measured although there is some doubt about its sensitivity. Table 2 shows a comparison of data for a single experiment for tail moment and tail length parameters as a function of X-irradiation. A more consistent response to increasing dose is seen with the tail moment parameter. 350 comets were analysed for each exposure group.

As tail moment is defined as the product of the tail length and the % of total DNA in the tail it is important to verify that reliable data for each of these components is acquired in the image analysis process. A major problem with the tail moment parameter is that it does not distinguish different shapes of the comet tail. Apart from DNA damage, there is a strong possibility that the experimental conditions can create changes in the comet tail. Factors that can significantly influence the result include the physical condition of the gel, the pH of the media, and inhomogeneities in the local electric field during electrophoresis. The accuracy of the tail moment is dependent on consistent shape of the comet tail. Tails with different lengths, number of strand breaks and relative amounts of DNA can have identical tail moments. By virtue of the process that essentially averages out the differences the tail moment may appear to give a more consistent value. As a result, the sensitivity of the tail moment value may be reduced.

Figure 13. Tail moment data for L5178Y S/S cells synchronised and irradiated in G1 with increasing doses of X-ray from 0.0 to 4.0 Gy.

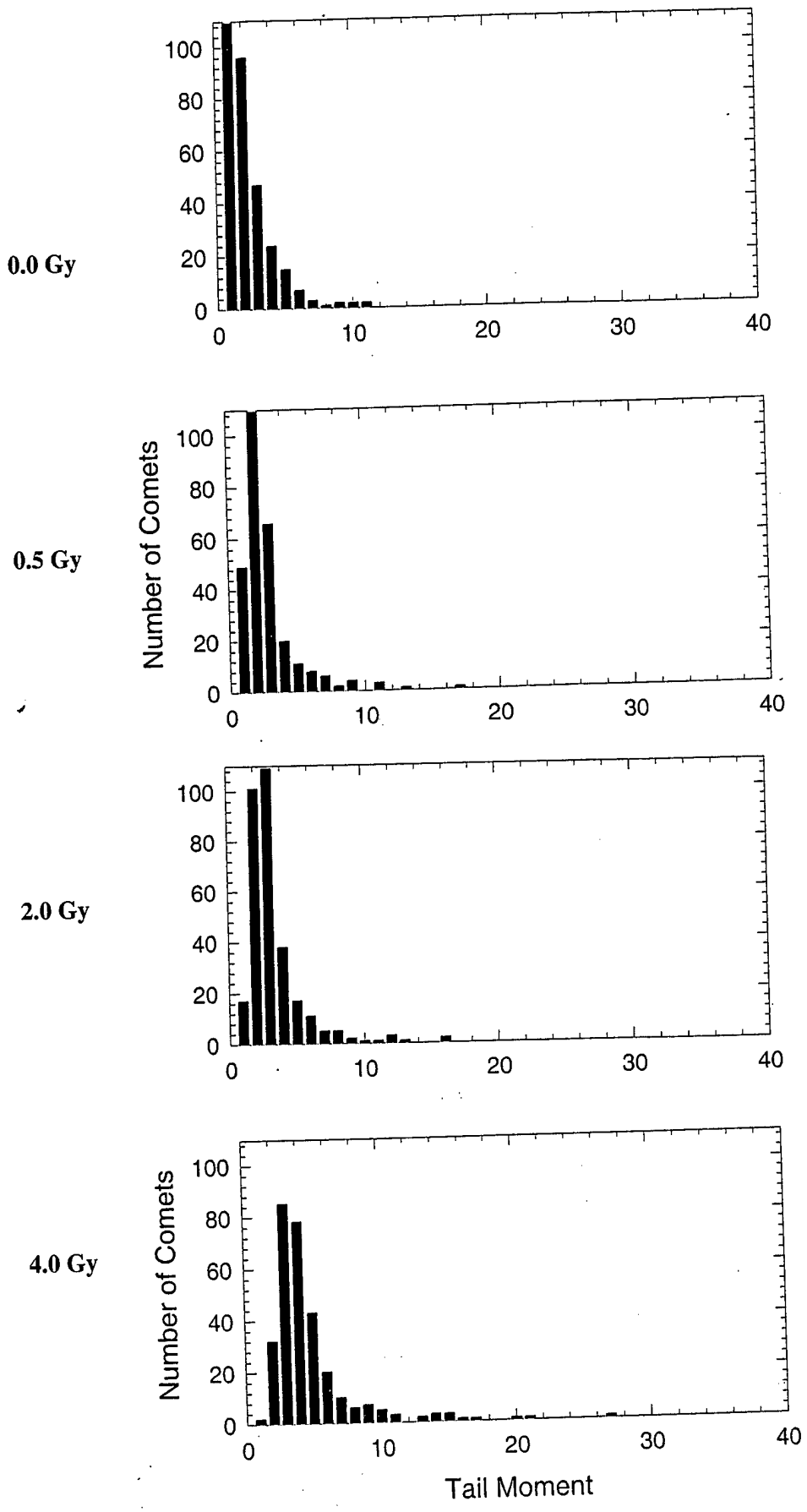

0.0 Gy
0.5 Gy
2.0 Gy
4.0 Gy
Number of Comets
Number of Comets
Tail Moment
0
20
40
60
80
100
0
10
20
30
40

Table 2. Comparison of mean values for tail length and tail moment comet assay parameters as a function of increasing X-irradiation in L5178Y S/S cells and unstimulated lymphocytes. (Data from a single experiment, n=350 comets per dose)

X-irradiation (Gy)	Tail Length	Tail Moment
L5178Y S/S		
0.0	11	0.64
0.5	14	1.49
1.0	12	1.63
3.0	14	2.33
4.0	17	2.65
Lymphocytes		
0.0	10	0.61
2.0	15	1.04

DNA Content

Data for DNA content (used in the calculation of tail moment) based on data from microscopic fluorescence was inconsistent with that obtained for DNA content using standardised cell flow cytometry techniques. Furthermore, the Coulter channeliser analysis of elutriation fractions confirmed that populations of cells with narrow size distributions around G1 were prepared for X-irradiation and comet assay. This was in agreement with the CFC evaluations. The analysis of DNA content from fluorescence was also internally inconsistent; there was wide variation for different samples of the same cell population. Some data appeared as two distinct populations with a wide range in mean DNA content (see Figure 14). In some cases this occurred in X-irradiated samples. There is no logical explanation for the wide range of values for tightly synchronised G1 cells, or for the apparent increase in DNA content in cells following exposure to X-irradiation.

There are a number of factors that contribute to variability in outcome and potentially abberrant results. DNA content is calculated from relative intensity of fluorescence between the comet and the background. This is directly influenced by the sensitivity of the ccd camera and the use of appropriate settings. Over-writing fluorescence will create a high background signal and reduce the sensitivity of detecting comets. On the other hand, the sensitivity must be sufficient to detect weaker signals from the edge of the comet tail. The staining procedure directly affects the extent of fluorescence, although this should be consistent for slides from the same population.

Figure 14. An example of the apparent change in DNA content in L5178Y S/S cells following exposure to X-irradiation.

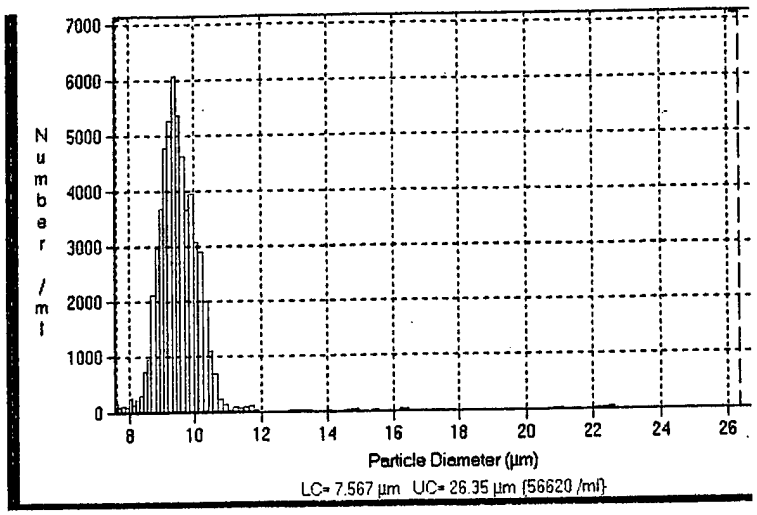
The graphs are from top to bottom;

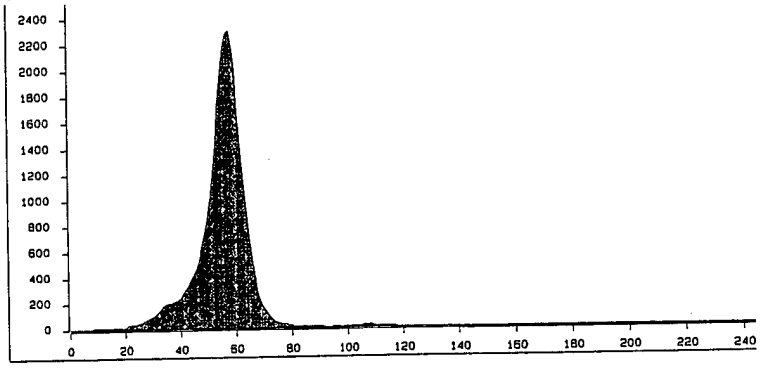
Elutriated G1 population size distribution,

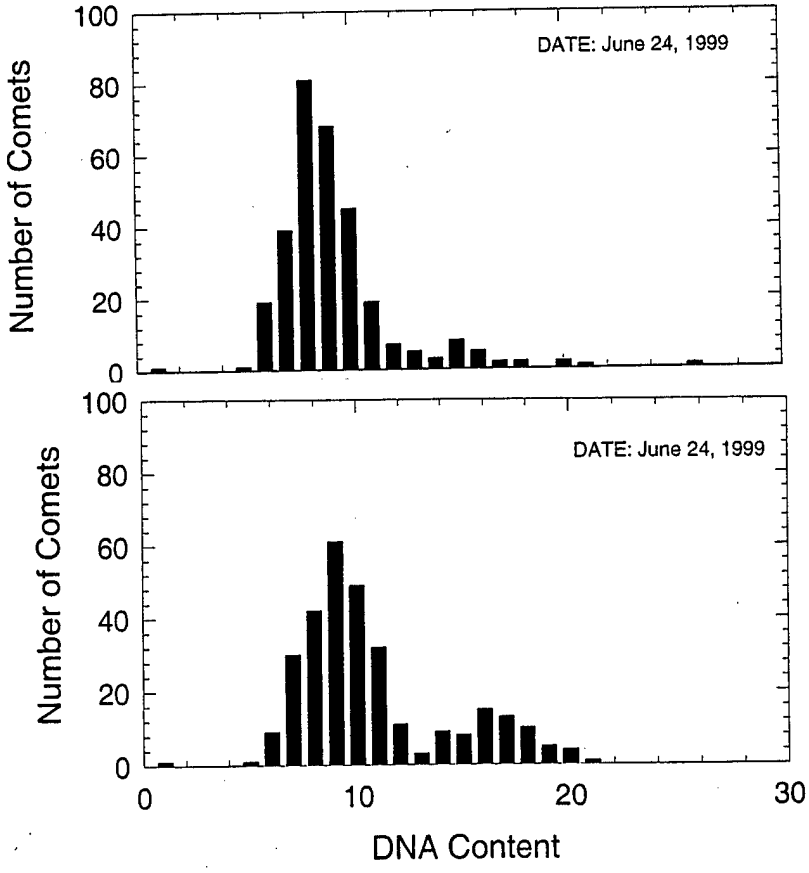
DNA content distribution from Cell Flow Cytometry analysis,

DNA content from comet assay in Zero dose control cells,

DNA content from comet assay in cells exposed to 4.0 Gy.


Number / ml
Particle Diameter (µm)
LC= 7.567 µm UC= 26.35 µm (56620 /ml)




DATE: June 24, 1999
Number of Comets
DATE: June 24, 1999
Number of Comets
DNA Content

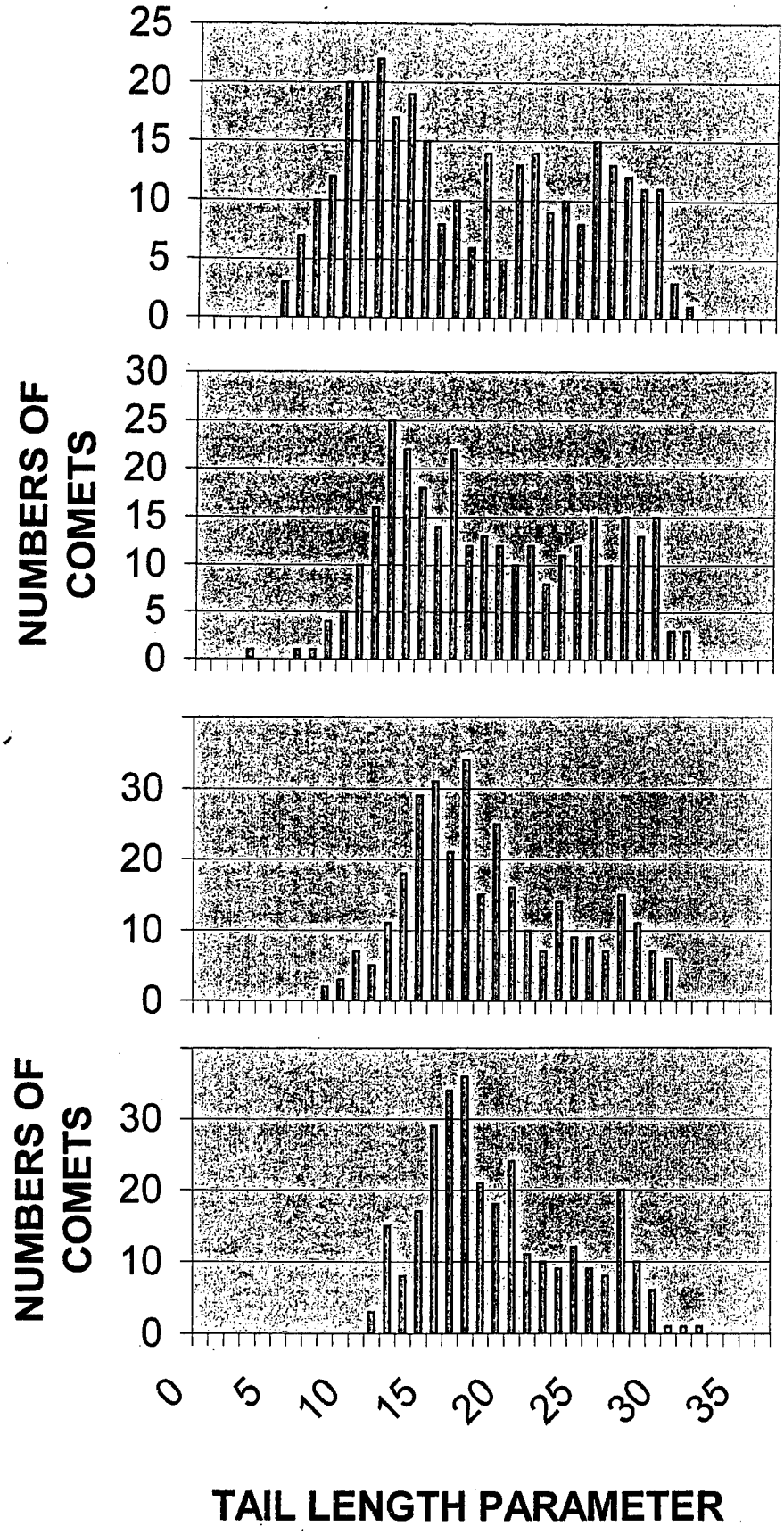
Analyses of DNA using the comet assay failed to show meaningful correlation with X-irradiation doses. Detailed examination also showed similar lack of correlation of results obtained using a normal asynchronous lymphocyte population. This problematic aspect of the comet assay is of concern. Examination of published scientific literature shows a remarkable absence of data on DNA content, despite the fact that this is an essential parameter in the calculation of either comet moment or tail moment. From private discussions with other researchers and from examination of the published literature on variations of the comet assay technique it is apparent that some do not fully understand DNA breakage and recombinant processes while others may simply choose to ignore an inconsistent parameter. As our study is the first to use synchronised G1 cell populations with the comet assay we have been able to clearly identify this important limitation by comparing data on DNA content obtained from the comet assay and from cell flow cytometry. We found a strong correlation between CFC data and our elutriation population profiles measured with the Coulter channeliser (see Figures 10, 11 and 14).

Tail length

Because of the inconsistencies in results for fluorescence-based DNA content, data were also analysed on the basis of tail length. Although this is a somewhat arbitrary measure these data are not subject to the range of variables that affect the other measures based on cell fluorescence. Examination of a recent publication by Olive *et al.* (1999) shows change from the original protocol using only tail moment to include data on tail length. Although tail length is given in arbitrary units the zero dose has a positive value of approximately 14. The value doubles with irradiation with 1 Gy and a dose response is reported up to 4 Gy. There are large variations in reported data; a standard deviation is shown of +/- 9 around the mean value 14 for 1 Gy irradiation. Tail moment values show a zero dose value closer to zero and a linear dose response to 30 Gy. Tail length is only a useful indicator at low doses (0-2 Gy) and rapidly reaches saturates above 2 Gy (Olive 1999). A recognised problem with the tail length parameter is that it gives limited information and cannot describe changes in comet tail shape or DNA content.

It is difficult to see how the tail length parameter can provide a sensitive indicator. Results of the present study (Figures 15-17) show that the inconsistency in data for tail length prevent sensible comparison of mean values for a range of X-ray doses. Figure 15 shows the response to X-irradiation of G1 synchronised L5178Y S/S cells measured by the comet assay derived tail length parameter.

Figure 15. Response to X-irradiation of G1 synchronised L5178Y S/S cells measured by the comet tail length parameter. (Increasing dose from the top of page downward; 0.0, 1.0, 2.0, 4.0 Gy.) A modest shift to the right is insignificant amongst the large variability in data. Note that the tail length distribution for control values covers a range of values by a factor of four.


25
20
15
10
5
0
30
25
20
15
10
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0
NUMBERS OF COMETS
30
20
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10
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NUMBERS OF COMETS
0
5
10
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20
25
30
35
TAIL LENGTH PARAMETER

Although it appears as though there is a weak correlation between the position of peak in tail length distribution and X-ray dose the wide spread of values for tail length make it impossible to achieve statistical significance. Tail length distribution for control values covers a range of values by a factor of four.

Evidence that there were significant problems in the comet image digital analysis is shown in Figure 15 where dog lymphocytes (G_0 cells) used as positive controls appear to have a bimodal distribution of tail length in control/unexposed blood. The distribution is remarkably similar to that of the tail lengths in the zero-dose controls of G1 samples of L5178Y S/S cells, both populations apparently having a bimodal distribution.

Data from asynchronous populations of L5178Y S/S cells also demonstrate the inconsistency of the tail length parameter. Plotting the mean tail length gives an apparent dose-response for low range, 0-1 Gy, but this disappears at higher doses of X-irradiation (Figure 17). The mean comet area parameter seemed to give a more definite dose-response over the range 0-3 Gy, but the standard deviation was approximately 25% of each mean value.

These findings supports the results of studies with unstimulated human lymphocytes (Bocher et al. 1997) which found the tail length to be appreciably less sensitive to X-ray doses than either the tail moment or head-to-tail ratio.

Figure 16. Response to X-irradiation of G1 synchronised L5178Y S/S cells and unstimulated G_0 lymphocytes measured by the comet tail length parameter.

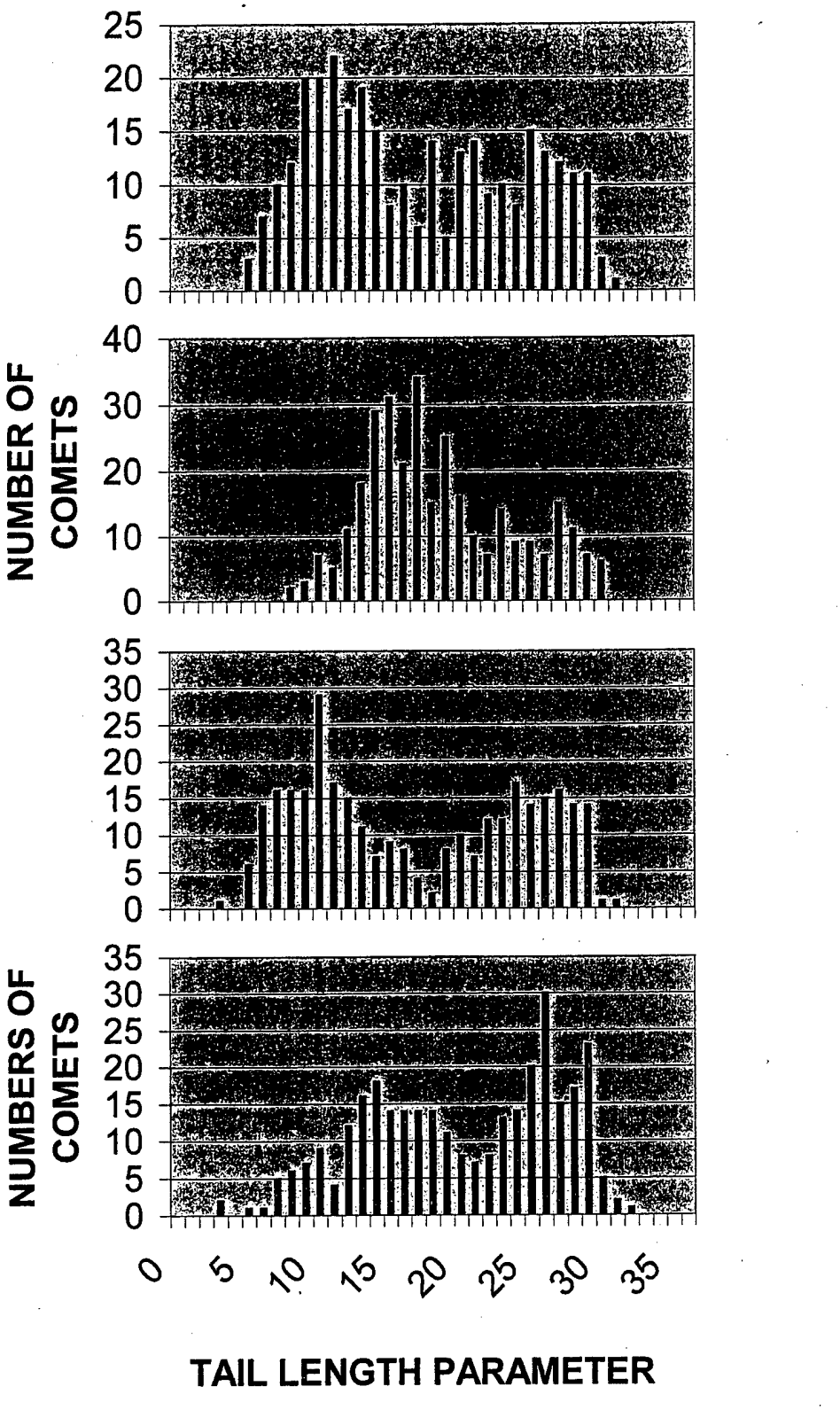
In order from top to bottom of page;

Zero dose L5178Y S/S cells,

2.0 Gy L5178Y S/S cells,

Zero dose Lymphocytes,

2.0 Gy Lymphocytes.


NUMBER OF COMETS
NUMBERS OF COMETS
TAIL LENGTH PARAMETER
0
5
10
15
20
25
30
35
40

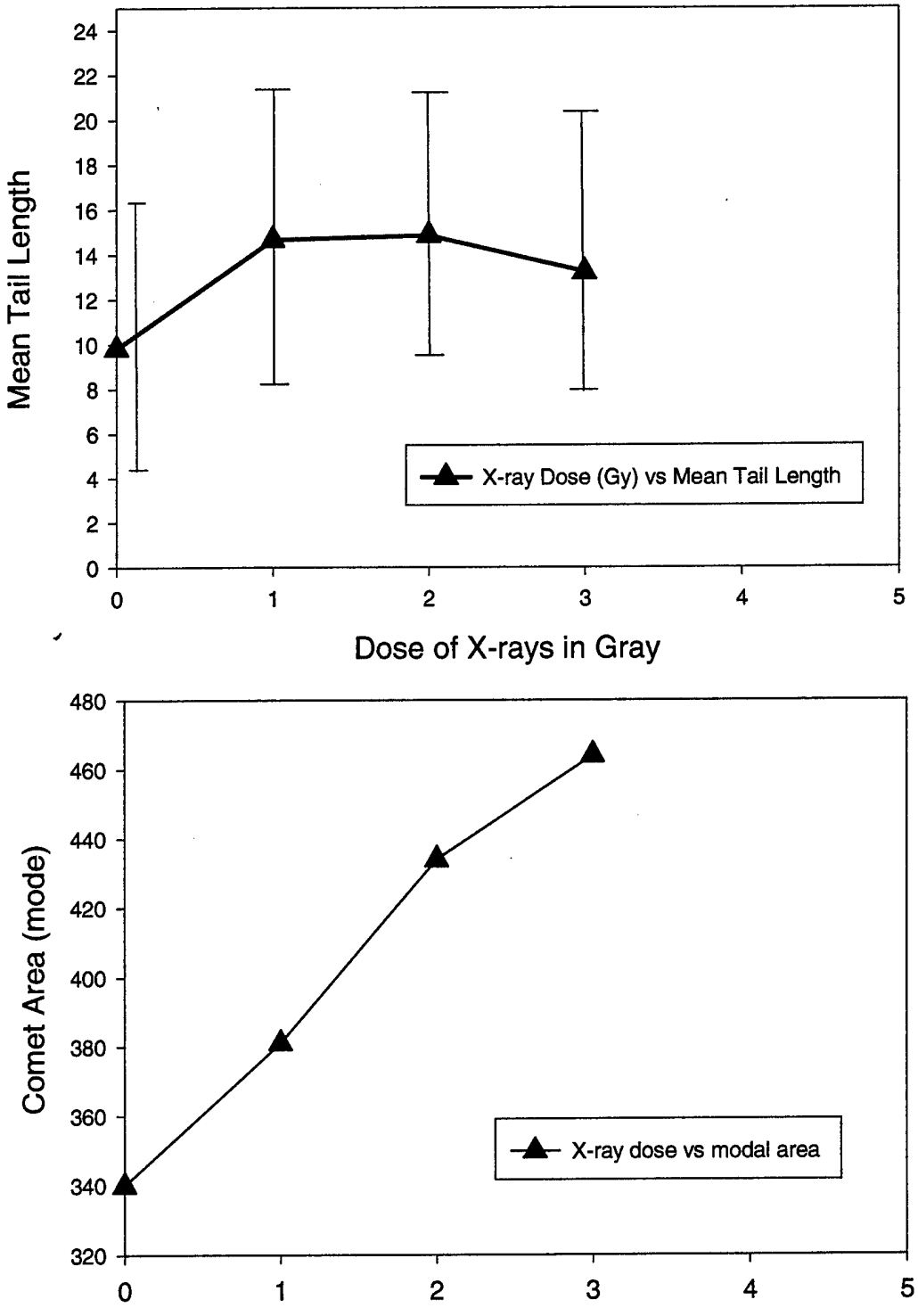


Figure 17 Mean tail length (upper) and comet area mode (lower) as a function of X-rays dose in Asynchronous L5178Y S/S cells.

Comet Assay Technical Problems

For reasons of economy the comet assay procedures were carried out in the Veterinary Teaching Hospital (VTH) of CSU where we had access to the original (Olive and Durand developed) equipment. The primary collaborative experimental work was carried out during visits by the principal investigator, of approximately six weeks in total, that coincided with availability of the VTH facility and staff and the elutriation facility at the Department of Radiological Health Sciences of CSU.

The requirements of this study to develop a sensitive radiation bioassay were found to be beyond the level of sensitivity normally used in routine procedures applied at the VTH, where the comet assay is used primarily to evaluate tumours. Although a considerable amount of effort was expended on modifying the experimental protocol, the results remained inconsistent. A range of technical difficulties arose during the series of experiments conducted for this study. Many of these were unexpected as the Principal Investigators had been assurred that the CSU facility held the expertise to undertake the comet assay. The situation was further aggrevated by continuing equipment breakdown.

A major problem with the comet assay technique is that the end product of DNA damage is derived from a computer based analysis and quantification of individual cell fluorescence in microscopic images. The degree of fluroscence is dependent on the staining and strand separation techniques and the sensitivity of the camera. Furthermore, criteria used to select images can be subjective. Therefore the preparation and image capture process can directly bias the result. In the present study the difficulties were compounded by continued equipment problems including breakdowns in the computer circuit board and the (ccd) camera used to capture the microscopic images. The computer

image capture board was repaired and problems with the ccd camera persisted throughout. Because of the high cost of this equipment total replacement was not an economic option.

A common technical problem that can cause problems for the comet assay, in general, is the variation in comet fluorescence (grey levels) in relation to the location of the comet. This remains a source of problems because of non-uniformity of illumination and of the response of the ccd camera/image intensifier. Bocher et al. (1997) identified these sources of error and proposed a mathematical image shading correction to remove camera and light source induced photometric nonlinearities.

The requirements of this collaborative study to develop a sensitive radiation bioassay were found to be beyond the level of sensitivity normally used in routine procedures applied at the VTH, where the comet assay is used primarily to evaluate tumours. Although a considerable amount of effort was expended on modifying the experimental protocol the final assay results remained unacceptably inconsistent. Many of the possible causes of these inconsistencies were outlined in the results section of this report.

Our findings are cause for some concern regarding the potential for increasing the sensitivity of the comet assay technique. This is a reflection on the standard, in general, of publications in the bioelectromagnetics field of research, in particular, where systematic and detailed study is seldom undertaken. Instead, it is common to see quick repeat studies of some reported (e.g., Lai and Singh 1995) positive effect of RF radiation with no attempt to understand the mechanisms involved or the reason for apparent discrepancies in results. Meanwhile, those who have invested substantial efforts into the comet assay demonstrate the inherent difficulties in the interpretation of data. Reporting on the reproducibility of the system after series of experiments over five years of research, Bocher et al. (1997)

commented that, "The results published in the literature have often been obtained with different methods for comet structure measurement. In most cases these data are not comparable with each other."

Our collaborative study also identified, apparently ignored data on inconsistency of data for DNA content; a fundamental parameter in calculating the tail moment. Hence, there may be good reason to doubt the reliability of data given in some publications on comet assays that do not disclose information on DNA content or valid zero-dose values. Our opinion was subsequently substantiated through private discussions with other scientists (Natarajan, Meltz, University of Texas Health Sciences Center) during the 6th Annual Michaelson Research Conference, August 1999. It is apparent that most researchers are either unaware of the limitation or choose to ignore it.

This international collaborative research project presented a range of technological challenges, most of which were successfully overcome. The apparent lack of sensitivity of the comet assay data was unexpected and the technical difficulties were unforseen. The investigators have agreed to continue to work, after submission of this report, with Dr. Sue Larue (VTH) to try to resolve some aspects of the alkaline hydrolysis technnique. It is now apparent that the technique is currently undergoing review by some experienced users who have spent many years refining the comet assay.

Results of our studies show that there is plenty of scope to increase the sensitivity of the assay, particularly for low dose radiation. The research partners are looking forward to the opportunity to continue to systematically examine and develop a useful and sensitive radiation bioassay.

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APPENDIX 1: *Flow Cytometry*

- For use with fluorescence microscopy (Zeiss Axioskop).

- Dilute cells in NIM (nuclear isolation medium) for counting. Place the amount of NIM, taken from the refrigerator, into a labelled plastic tube. Vortex the cells in 15 ml centrifuge tube, then place the appropriate amount of cells in the plastic tube with the NIM.

Vortex the counting dilution. Choose the, dilution based on estimation of cell density.

990 µl NIM + 10 µl cells = 1:100 dilution
974 µl NIM + 26 µl cells = 1:40 dilution
950 µl NIM + 50 µl cells = 1.20 dilution
450 µl NIM + 50 µl cells = 1:10 dilution

** Minimize the time the dilution is exposed to fluorescent light because the NIM contains a fluorescent dye.

- Using a capillary tube, fill the haemocytometer with the counting dilution. Place on stage of fluorescence microscope. Push in the filter lever to obtain a green filter (appears red when looking through the scope eyepieces). Count the 5x5 chamber on both sides of the haemocytometer and average the counts. The cells have a red-orange, round appearance.

Multiply the average count by the dilution and haemocytometer factors.

example: 1:40 dilution in NIM factor
1:10,000 haemocytometer factor average of 30 cells
40 x 10,000 x 30 = 12,000,000 or $12x10^6$ cells/ml

- Number of cells needed for fixation sample:

BrdU:	$2.4x10^6$ cells/tube
cells only	$2.0x10^6$ cells/tube

Place the appropriate volume from the cell suspension into a centrifuge tube:

no. cells needed/no. cells counted = volume of cell suspension (mls.) in tube

eg: $2x10^6 / 12x10^6 = 0.167ml$

Cell Fixation

Centrifuge L5178Y S/S cell suspension at 1100 rpm for 5 min. Resuspend $2x10^6$ cells in refrigerated CABS (citric acid buffer solution) to the tube to equal 2 ml. While vortexing, slowly (one drop quickly after another) add 2 ml of Absolute ethanol from the freezer. Place the cap on the tube containing 4 ml sample, confirm correct labelling and place in a styrofoam rack in the refrigerator.

CABS;

Sucrose	85.3 gm
trisodium citrate	11.8 gm
dimethyl sulphoxide (DMSO)	50.0 ml

Dissolve sucrose and citrate in distilled water

Add DMSO, add H_2O to 1,000 ml final volume.

Adjust to pH7.6.

APPENDIX 2: *Comet Assay Protocol*

The comet assay experimental protocol was adapted from that of Olive *et al.* for use with L5178Y S/S cells.

Gel and Slide Preparation:

Dissolve low gelling temperature agarose to make a 1% solution in phosphate buffered saline. Heat to approx. 80°C (keep below boiling) and cool to 40°C in a water bath. Precoat microscope slides (preferably use half frosted type) by pipetting 50 µl agarose and spreading over slide surface. Allow to dry to a thin film.

Single Cell Gel preparation:

Mix 0.5 ml cell suspension with 1.5 ml agarose by pipetting up and down a few times. Rapidly pipette 1.5 mls agarose/cell mix onto a slide to a final density of $1x10^4$ cells. Allow 1 - 2 min for agarose to gel on slide.

Protein lysis in high salt/detergent:

Submerge slides in freshly prepared lysis buffer;

0.03 M NaOH

1.2 M NaCl

0.5% N-lauroylsarcosine

Lyse slides in the dark at room temperature for 1 hr. This may be modified to lyse in the dark in the refrigerator at 4°C for up to 24 hr. Lysis time affects the amount of DNA unwinding.

Lysis is carried out with the slides arranged horizontally in a wide container. As a result of the large surface area pH can change substantially with time. Therefore, it is essential to always use freshly prepared lysis solution and keep the container covered.

Denature DNA in alkali:

Quickly transfer slides to avoid renature of DNA into an alkali rinse solution;

0.03 M NaOH

2 mM EDTA

This rinse removes the NaCl which is essential for lysis but which substantially reduces DNA migration during the subsequent electrophoresis. Salt will also alter the current as it diffuses out of the gel. (This can cause inconsistent results)

Rinse slides for 1 hr total in 2 – 3 changes of alkali rinse solution.

Electrophoresis:

Place slides in horizontal gel electrophoresis chamber in a fresh solution of alkali rinse so that the surface of the agarose gel is just covered.

Electrophorese for 25 min at 0.5 V/cm. The current (~130 mA) should not vary significantly during electrophoresis.

Staining:

Rinse slides in DDW for a few mins. Stain for 20 min in 2.5 μg/ml propidium iodide dissolved in 0.1M NaCl. Rinse slides in DDW and place on wet paper towels in air and light-tight boxes. Store in the refrigerator.

Comet assay equipment requirements:

Computer-controlled data acquisition, SCG electrophoresis, Zeiss fluorescence microscope, ccd camera, image intensifier, high resolution graphics monitor, custom-written image analysis software, data analysis software (sigma-plot). X-ray source for positive controls.

APPENDIX 3: *Lymphocyte Preparation*

1. Collect 10 ml of whole blood (from normal dog) into a green top heparinised blood tube. Gently shake and keep at room temperature.

2. Transfer to a 15ml centrifuge tube and add 10 ml PBS to the blood and gently mix (make a 1:1 dilution).

3. Place 3 ml of density 1.077 Ficoll-hypaque (Histopaque, Sigma) into each of two 15 ml centrifuge tubes and allow the gradient to come to room temperature. Gently layer the diluted blood onto the Histopaque by holding the tube at an angle and slowly pipetting the blood down the side of the tube. Do not mix the blood and Histopaque.

4. Centrifuge on high speed for 20 min. Using a transfer pipette remove the lymphocyte band at the gradient interface, trying to avoid collecting any of the Histopaque.

5. Place the lymphocytes in a labelled centrifuge tube with 10 ml PBS and wash. If the Histopaque is not completely diluted and washed out lymphocytes will remain in suspension and cells will be lost during the procedure.

6. Centrifuge 5 min. at setting #4. Decant and resuspend in PBS to 1 ml. Refrigerate until ready to count.

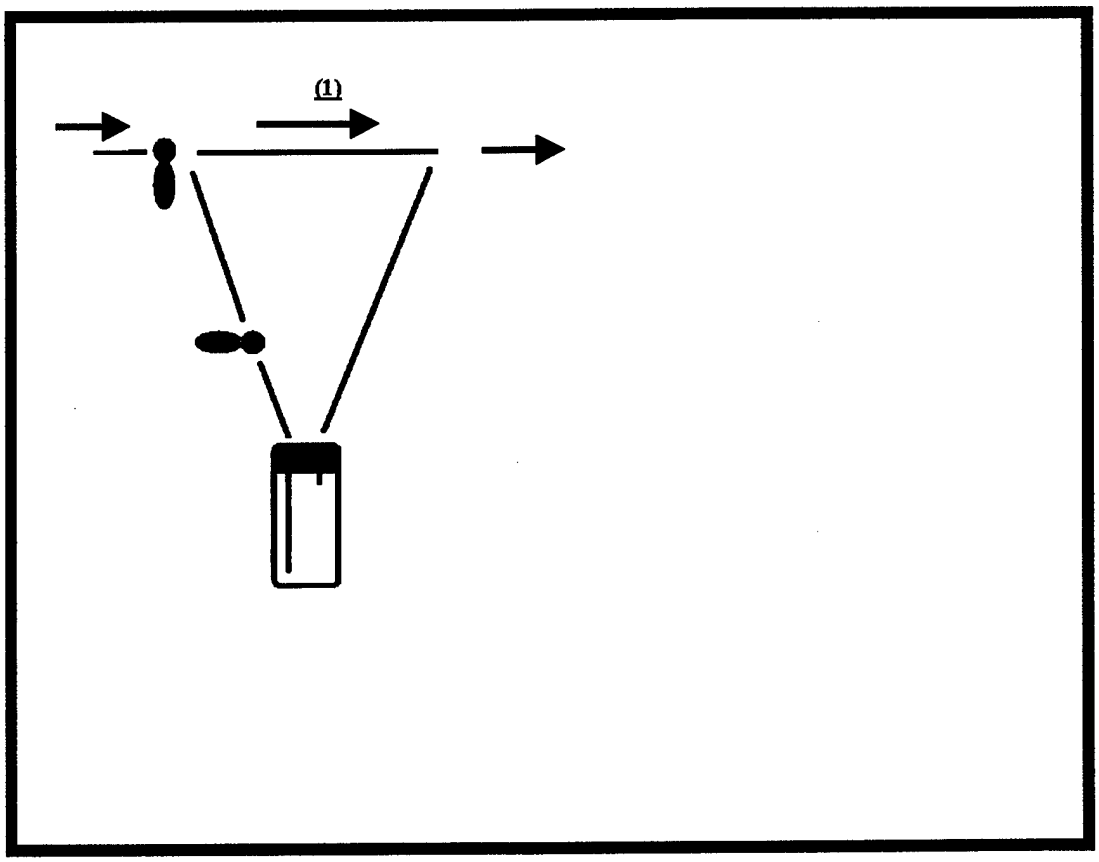
7. When ready to count, break up cells by aspirating through a 3 1/2" x 22 gauge spinal needle 2-3 times. Discharge suspension into a clean centrifuge tube.

8. Follow procedures outlined in "Counting and Fixing Cells".

APPENDIX 4:A: - *Elutriation Preparation*

(1) Purge Air from System:

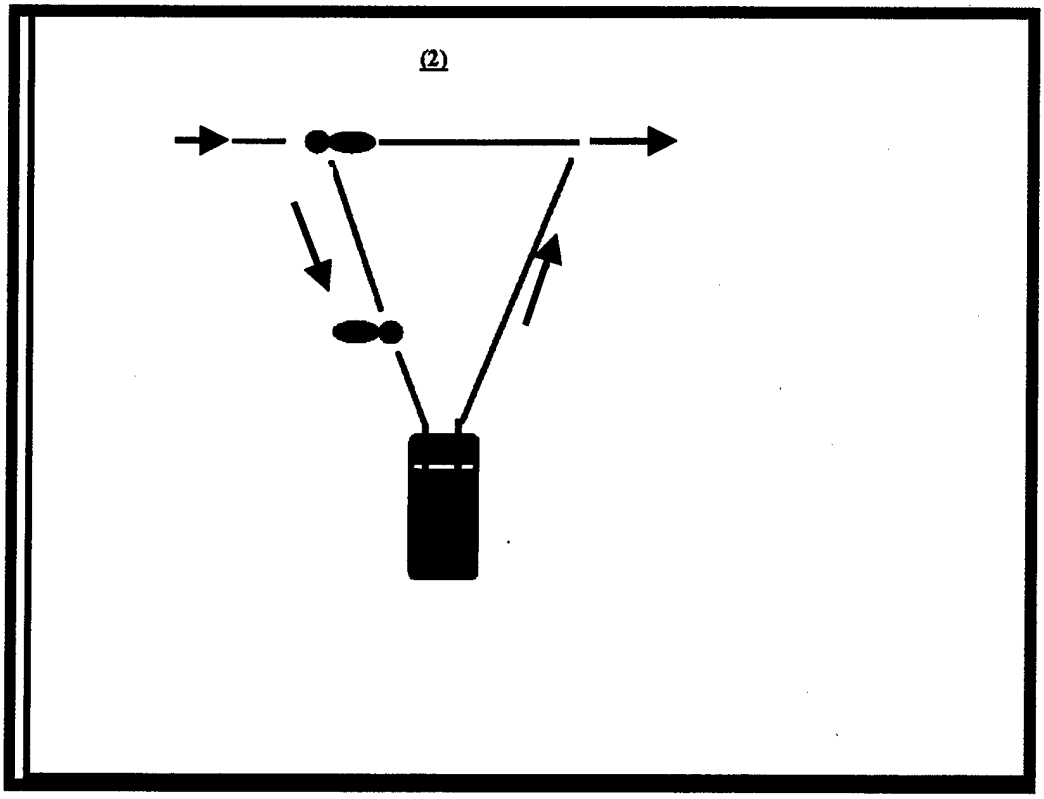
- Set Peristaltic Pump @ 20 ml/min and Rotor Speed @ 1000 rpm
- Bypass Valve set at 6 o'clock
- Clamp tubing downstream of pressure gauge to 10 psi, release & discharge bubbles
- Check Rotor for bubbles
- Increase rotor speed to 2550 rpm
- Watch line pressure, - if increases above 3-4 psi, stop rotor (this usually allows bubbles to leave rotor)
- Restart at 2550 rpm



APPENDIX 4:A: - *Elutriation Preparation*

(2) Fill harness and Mixing Chamber with liquid:

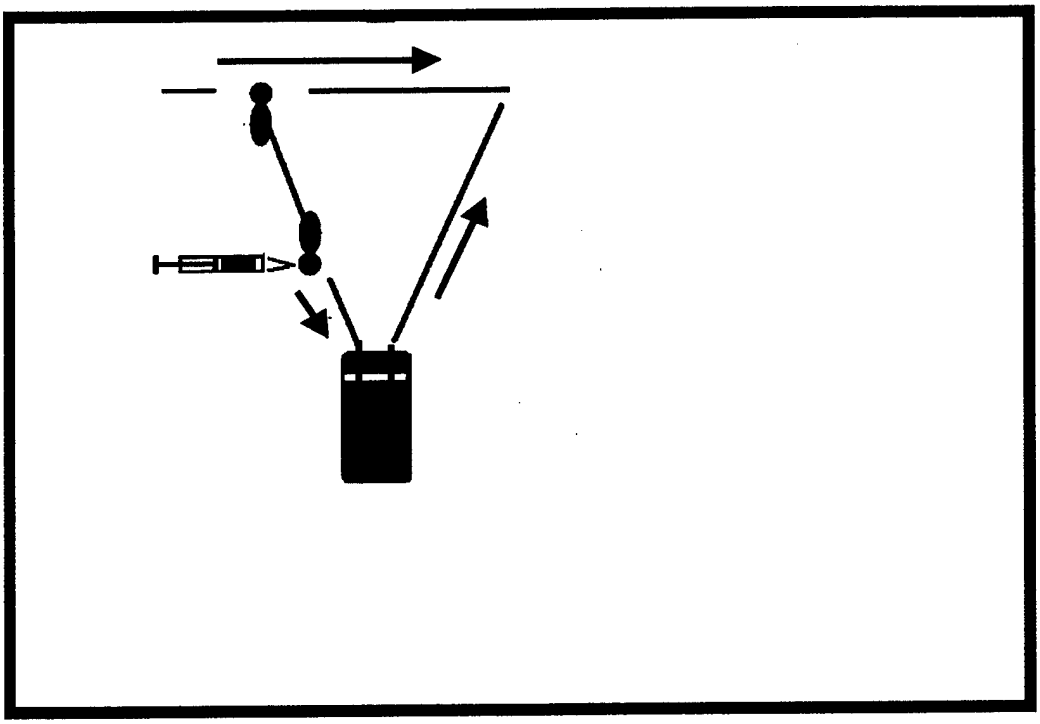
- Push in short needle to make bubble trap
- Turn Bypass Valve to 3 o'clock
- Injection Valve set at 9 o'clock
- Check for bubbles in lines and remove if necessary



APPENDIX 4:A: - *Elutriation Preparation*

(3) Inject Cell Sample in Mixing Chamber:

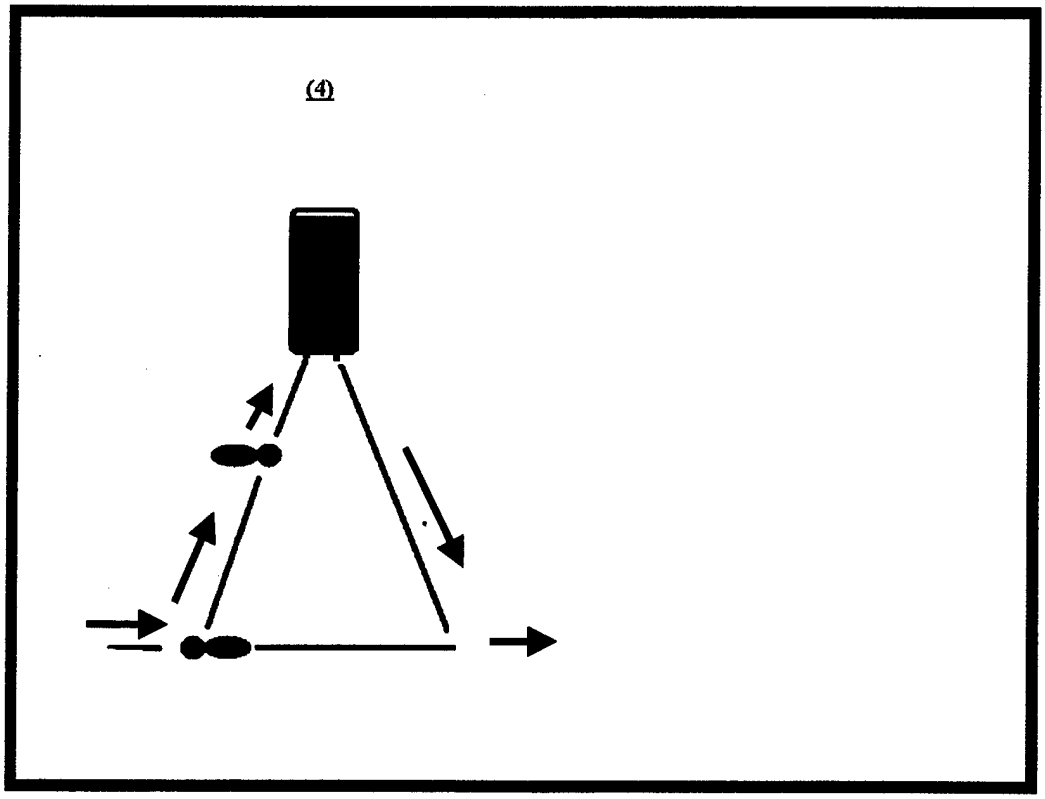
- Turn Bypass Valve to 6 o'clock
- Connect 50 ml plastic syringe by Luer-lock
- Turn Injection Valve to 12 o'clock (beware of back-pressure)
- Inject 20 ml cell suspension ~$1x10^8$ cells, slowly ~ 60 sec (back pressure <2 psi)
- Turn Injection Valve to 9 o'clock
- Remove syringe
- Check rotor & pump speed



APPENDIX 4:A: - *Elutriation Preparation*

(4) Deliver Cells to Rotor:

- Invert Mixing Chamber
- Turn Bypass Valve to 3 o'clock
- Fix injection chamber in inverted position and allow media to flow through during entire elutriation, - acts as large capacity bubble trap
- Visually check cell collection in Rotor – if too strong a boundary persists through collections there is probably mixing in the rotor elutriation chamber or transfer tube and poor fractions will result.



APPENDIX 4:B: - *Elutriate Cell Fractions By Size*

1) The in-line pressure gauge is used to constantly monitor back-pressure from the rotor. Cell fractions are collected in volumes of 50 ml.

2) The 1st fraction is collected during injection of the sample with rotor speed set at 2550 rpm and culture medium fluid flow rate set at 20 ml/min. Three more fractions are taken while the rotor speed is at 2550 rpm.

3) As soon as the 4th fraction is collected, slowly decelerate the rotor 2350 rpm using the fine Vernier-graduated control. Avoid quick deceleration which can cause the rotor brake to temporarily reduce speed too far and mix the cell fractions. Collect four fractions.

4) As soon as the 4th fraction has been collected, decelerate the rotor to 2220 rpm and immediately as the decceleration begins collect the next four 50 ml fractions. While collecting these samples, check the size distributions and numbers of cells for desired values. Small, necessary changes in rotor speed can be made at this stage.

5) Repeat the previous stage after reducing the rotor speed to 1980 rpm. Collect four fractions.

6) Reduce the rotor speed to 1750 rpm and collect two fractions.

7) Reduce the rotor speed to 0 rpm and collect two fractions.

8) After the desired populations are collected, flush out and dispose of the remaining cells by turning off the rotor drive and allowing the rotor to come to a complete stop.

9) Turn off the fluid couterflow, change the fluid line back to sterile water, accelerate the rotor to 1000 r.p.m., and pump at least 500 ml through the system to flush out the medium. In order to adequately flush the system the rotor must be operating. Bring the rotor to a full stop.

10) Dismantle the rotor, wash with deionized water and dry in air.